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Differential Responsiveness of A and C3H Mouse Mammary Tissues to Somatotropin-Containing Hormone Combinations.* (26826)

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Somatotropin has previously been reported to act as a lobuloalveolar mammogen and as a lactogen in the C3H/Crgl strain of mice, when administered along with appropriate ovarian hormones and adrenocortical hormones, respectively(1). In the C3H/Crgl strain, bovine somatotropin and ovine mammotropin were found to be interchangeable in the experimental production of mammogenesis as well as of lactogenesis. The lactogenic effect of somatotropin had not been reported in any other species; accordingly, we determined the mammary responsiveness of various other inbred strains of mice to somatotropin-containing and mammotropin-containing hormone combinations. Treatment with estradiol + progesterone + mammotropin followed by cortisol + mammotropin was effective in inducing milk secretion in the mammary glands of hypophysectomized-ovariectomized female mice of the 10 inbred strains and sublines tested(2). Estradiol + progesterone + somatotropin followed by cortisol + somatotropin initiated milk secretion in all except the A strains of mice.

The A/Crgl and C3H/Crgl strains both carry the mammary tumor agent and both have a high incidence of spontaneous mammary tumors(2). The high mammary tumor incidence in A/Crgl mice is restricted, however, to breeding females, whereas the incidence is high in virgin as well as in breeding females of the C3H/Crgl strain. The difference in mammary gland response to the somatotropin-containing hormone combination may be due to a difference in the genetic constitution of the 2 strains resulting in (a) a difference in rate of metabolism and/or excretion of the administered hormone or (b) a difference in responsiveness of the mammary tissue itself. The present investigation was undertaken to distinguish between these two possibilities.

Materials and methods. A/Crgl and C3H/Crgl mice and their reciprocal hybrids (A ×

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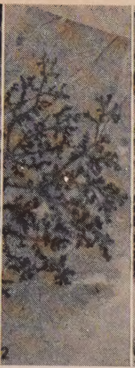
C3H, C3H × A) were used. The growing mammary gland parenchyma, occupying only a small portion of the inguinal (#4) mammary fat pads of 3-week-old hybrids, was surgically removed. Approximately 1 mm^3 pieces of primary ducts from donor A and C3H female mice were immediately transplanted into these mammary gland-free fat pads by a technic described previously(3). Each hybrid host received one C3H transplant in one fat pad and one A transplant in the contralateral fat pad. Three-week-old females, 9-month-old virgin females, and 4-month-old nulliparous pregnant females at the 12th to 14th day of pregnancy were used as donors; tissues transplanted into a given host animal were taken from donors of the same age. In this manner, genetically different mammary tissues of similar age and physiological state could be followed under identical hormonal conditions in the same host. The host mice were hypophysectomized-ovariectomized at the age of 5 weeks, and treated beginning on the first post-operative day with estradiol-17β (E) + progesterone (P) + somatotropin (STH) for 21 days to induce lobuloalveolar development. This regimen was followed by injection of cortisol acetate (F) + STH for 5 days to initiate milk secretion. All hormones were administered as daily subcutaneous injections of aqueous suspension (steroids) or solution (STH). The steroids E and P were combined in a single injection. Daily doses of E, P, and F were 1 μg, 1 mg, and 125 μg, respectively. The STH was prepared from bovine pituitaries according to the method of Li(4) and was reported to contain approximately 0.5% mammotropin and less than 1% of other pituitary hormones. Animals were treated with increasing doses of STH as follows: 100 μg (days 1-7), 500 μg (days 8-14), and 1000 μg (days 15-26). Half of the 8-10 animals in each experimental group were killed on the 22nd day to study the extent of lobuloalveolar growth; the other half were terminated on the 27th day to determine lactogenic response. At autopsy, the mammary glands were prepared as whole-mounts and stained with hematoxylin. The state of alveolar development was determined from study of stained whole-mount preparation. Presence or absence of milk secretion was ascertained by examination of exposed mammary glands prior to fixation.

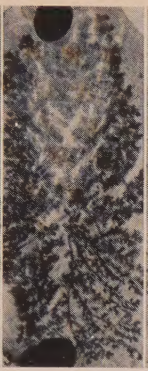
Results and discussion. Significant differences in lobuloalveolar and lactogenic response to the hormone treatment were observed between the 2 types of transplanted tissue, and between transplants and host (hybrid) mammary tissue. Among the C3H transplants used, response of mammary tissues from 4-month-old nulliparous pregnant females was slightly greater than that from virgin females. However, no appreciable differences were observed in the response of transplants from A donors in spite of variations in age or physiological state.

Treatment with E + P + STH resulted in development of some alveoli and a few lobules in the hybrid mammary glands. The transplants from A donors grew to occupy 30 to 100% of the fat pad, and consisted generally of ducts, many end buds, a few isolated alveoli, and occasional small lobules (Fig. 1). The amount of growth seen in C3H transplants was similar to that seen in A transplants, but there were many more lobules in the mammary parenchyma of the C3H transplants than in either A transplants or hybrid mammary glands.

Treatment with E + P + STH followed by F + STH induced occasional milk secretion in parts of the mammary glands of a few hybrid hosts (Fig. 2). This same treatment initiated milk secretion in most of the C3H transplants but in none of the A transplants. The majority of the C3H transplants had dilated lobules filled with milk (Fig. 3). The mammary parenchyma of the A transplants consisted primarily of ducts and regressed alveoli (Fig. 4), although occasional lobules were maintained.

These results clearly demonstrate that the difference in mammary responsiveness of A/Crgl and C3H/Crgl mice to the somatotropin-containing hormone combination is due to the difference in genetic make-up of the mammary tissues, and cannot be explained on the basis of differences in rate of metabolism and/or excretion of hormones in the 2 strains. Gardner and Argyris(5) came to a





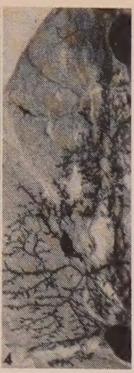


FIG. 1. Outgrowth from a piece of mammary duct from a 4-mo-old nulliparous pregnant A mouse transplanted into a female A × C3H hybrid. Host hypophysectomized-ovariectomized at 5 wk of age and treated with estradiol + progesterone + somatotropin for 21 days. Note presence of a few alveoli. Hematoxylin. × 3.5.

FIG. 2. Host gland from A × C3H female, hypophysectomized-ovariectomized at 5 wk of age and treated with estradiol + progesterone + somatotropin for 21 days followed by cortisol + somatotropin for 5 days. Note presence of some milk-filled alveoli. Hematoxylin. × 5.

FIG. 3. Outgrowth from a piece of mammary duct from a 4-mo-old nulliparous pregnant C3H female, transplanted into the same hybrid host described in Fig. 2. Note presence of large number of milk-filled lobules. Hematoxylin. × 3.5.

FIG. 4. Outgrowth from a piece of mammary duct from a 4-mo-old nulliparous pregnant A mouse transplanted into the same hybrid host described in Fig. 2. Note absence of milk-filled alveoli. Hematoxylin. × 3.5.

similar conclusion in their studies of the strain differences in estrogen sensitivity of the vaginal mucosa of mice.

Summary and conclusions. Mammary tissues from A/Crgl and C3H/Crgl strains of mice were transplanted into mammary gland-free fat pads of female hybrids (C3H × A, A × C3H). The hosts were hypophysectomized-ovariectomized, and treated with estradiol + progesterone + somatotropin for 21 days. Half of them received an additional 5 days treatment with cortisol + somatotropin. On the basis of the lobuloalveolar mammogenic and lactogenic responsiveness of the A, C3H, and hybrid mammary tissues to the hormone combinations used, the following general conclusions were drawn: 1. There is a quantitative difference in lobuloalveolar mammogenic response of A/Crgl and C3H/Crgl mammary tissues to the somatotropin-containing hormone combination, degree of response being greater in the C3H/Crgl mammary gland. 2. There appears to be a qualitative difference in lactogenic response of A and C3H mammary tissue to the somatotropin-containing combination. 3. The occurrence of milk secretion in mammary glands of hybrids (A × C3H, C3H × A) and in C3H mammary transplants, and its absence from A transplants, indicate that the genetic determinant for mammary gland sensitivity to the somatotropin-containing hormone combination must lie in the mammary tissue itself, and that this can be transmitted by either male or female C3H parent to the hybrid offspring.

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Effect of Enol Etherification on Antiestrual and Contraceptive Activity of Some Progestins in Rats. (26827)

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A series of several enol ethers of Δ^4-3-ketosteroid hormones recently synthesized in our laboratories(1) has shown more potent hormonal activity when administered orally than their respective parent compounds(2-4). Enhancement of the activity was dependent upon the nature of the alcohol chain. The present investigation was designed to study

TABLE I. Influence of Progesterone on Estrous Cycle of Rats.

Compound	Dose/animal/day mg	Dose/animal/day µmoles	No. of rats with estrous cycle* Maintained	No. of rats with estrous cycle* Inhibited	Length of cycle (days)
Sesame oil	—	—	10	0	4.0 ± .2†
Progesterone	.628	2	10	0	5.9 ± .2
	1.256	4	10	0	12.8 ± 2.3
	2.512	8	0	10	—

* 10 animals/group.

† Mean ± stand. error.

the change in activity produced by enol etherification of some progestational steroid compounds on the estrous cycles of rats. The best results in earlier experiments were obtained using cyclopentyl derivatives of 17α-methyltestosterone(2,3) and 17α-acetoxyprogesterone(4); therefore, cyclopentyl enol ether derivatives were also used for these experiments.

Materials and methods. The experimental animals were laboratory-bred, albino rats, weighing 160-180 g, fed a normal laboratory diet with unrestricted access to water. The females were selected at random, marked, and distributed 5 to a cage. Their vaginal smears were studied for 2 to 3 weeks in order to ascertain the regularity of their estrous cycles; female rats showing irregular cycles were discarded. On the 16th day of steroid treatment, 2 males were placed in each cage of 5 females and were withdrawn after 10 days of cohabitation. Steroid treatment was terminated on the 31st day, and the females were observed until the 50th day, in order to allow the pregnant ones to reach delivery time. Daily vaginal smears were obtained with a suitable pipette after lubricating the vagina with a drop of saline. This procedure was used to minimize trauma. The smears were stained with methylene blue; cornified epithelial cells without leucocytes were regarded as evidence of estrus, and the presence of spermatozoa was considered an indication of mating. The steroids used were the following: progesterone, 17α-acetoxyprogesterone (AP), 6α-methyl-17-α-acetoxyprogesterone (MAP), 17α-ethynyl-19-nortestosterone acetate (ENTA), and the cyclopentyl enol ethers of the 3 latter compounds (APc-5, MAPc-5 and ENTAc-5 respectively).*

The progestins were dissolved in 0.2 cc of sesame oil and administered daily by gastric tube, with the exception of progesterone which was given subcutaneously.

Results and discussion. Table I shows the effect of graded doses of progesterone on the estrous cycle of the rat. At a dose of 2 µmoles/animal/day there was a slight prolongation of the interval between 2 estrus-positive smears; increasing delay was obtained using 4 µmoles/day, and complete inhibition with 8 µmoles/day. These results, showing that diestrus can be lengthened in proportion to amount of progesterone administered, are essentially in agreement with the findings reported by others(5-8).

Table II compares the effects of AP, MAP, and ENTA with those of their respective cyclopentyl enol ethers. Neither AP at 1-2 µmoles/day, nor its enol ether derivative even at 20 µmoles/day, showed any inhibitory effect on the estrous cycle, mating, or fertilization confirming earlier findings(4) that AP cyclopentyl enol ether was endowed with strong oral progestational activity but devoid of contraceptive effects. On the other hand, MAP proved to be a potent inhibitor of estrus in the rat. At 0.5 µmole/day more than half of the animals showed inhibition of the cycle; in the remaining ones, there was a marked prolongation of the cycle. At 1 µmole/day all the females failed to have their estrus. Furthermore, both dosage levels of MAP prevented mating. Thus the results, reported by Sala *et al.*(9) on subcutaneous administration of MAP, can be obtained also by oral administration. Interestingly, enol etherification of MAP with cyclopentylic al-

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* The physical constants of these 3 compounds are: APc-5, m.p. 158-159.5°, $[a]_D = -142°$; MAPc-5, m.p. 169-171°, $[a]_D = -172°$; ENTAc-5, m.p. 181-183°, $[a]_D = -215°$.

TABLE II. Influence of 17α-acetoxyprogesterone (AP), 6α-methyl-17α-acetoxyprogesterone (MAP), 17α-ethynyl-19-nortestosterone acetate (ENTA) and of Their Cyclopentyl Enol Ethers on Estrous Cycle, Mating and Fertilization of Female Rats.

	Dose/animal per day		No. of rats with estrous cycle*				No. of matings per rat in 10 days		
Compound	mg	µmoles	Maintained	Inhibited	Length of cycle (days)†	No. rats mated	Mean	Range	No. of deliveries
Sesame oil	—	—	10	0	4.0 ± .1‡	10	1.1	(1-2)	10
AP	.372	1	10	0	4.6 ± .4	9	.9	(0-1)	7
	.745	2	10	0	4.7 ± .2	9	.9	(0-1)	8
APc-5	.881	2	10	0	4.6 ± .4	10	1.0	(1-1)	8
	4.406	10	10	0	4.6 ± .4	10	1.2	(1-2)	9
	8.812	20	10	0	5.1 ± .3	10	1.3	(1-2)	10
MAP	.193	.5	4	6	7.2 ±2.3	2	.2	(0-1)	0
	.387	1.0	0	10	—	0	.0	(0-0)	0
MAPc-5	.227	.5	10	0	4.3 ± .1	9	1.0	(0-2)	8
	.455	1	10	0	4.3 ± .1	10	1.0	(1-1)	10
	.909	2	10	0	4.5 ± .3	10	1.0	(1-1)	10
	1.819	4	5	5	4.6 ± .5	6	.6	(0-1)	6
	3.637	8	0	10	—	9	1.0	(0-2)	7
ENTA	.170	.5	10	0	4.0 ± .1	10	1.3	(1-2)	7
	.340	1	10	0	4.3 ± .3	10	1.1	(1-2)	9
	.681	2	10	0	4.8 ± .3	10	1.1	(1-2)	4
	1.362	4	10	0	6.9 ±1.4	9	1.1	(0-3)	0
	2.724	8	0	10	—	9	2.2	(0-5)	0
ENTAc-5	.204	.5	10	0	4.4 ± .3	9	1.0	(0-2)	3
	.409	1	10	0	4.0 ± .4	9	1.3	(0-2)	0
	.817	2	1	9	9.0	10	2.4	(1-5)	0
	1.634	4	0	10	—	9	2.4	(0-5)	0
	3.268	8	0	10	—	8	2.1	(0-5)	0

* 10 animals/group. † In the animals showing estrus. ‡ Mean ± stand. error.

cohol brought about a marked reduction of its previously described anti-estrual activity—a dose of 8 µmoles of MAPc-5 was necessary to inhibit estrus in all the animals. It is noteworthy, however, that at lower dosage levels the cycle was apparently not prolonged; the doses which inhibited the cycle did not interfere with copulation and pregnancy in most cases. Thus it can be assumed that the only action of MAPc-5 was to inhibit epithelial cornification of the vagina, without affecting the occurrence of 'heat' (considered as willingness to mate), ovulation and fertilization. However, we know that sexual receptivity has been reported occasionally in old rats(10) or in rats likely to suffer from abnormalities of the reproductive tract(11) or in x-irradiated animals(12) at times other than typical 'estrus'.

ENTA and its derivative ENTAc-5 had different effects than the other progestins reported here. Table II shows that 2 µmoles of ENTA/day adversely affected ovulation and fertilization, although neither estrus nor mating was affected. Inhibition of estrus increased with dosage level—4 µmoles prolonged the cycle, and 8 µmoles inhibited it completely. These high dosages of ENTA did not hinder mating; in fact, in some of the animals mating was more frequent than expected. Treatment with ENTA, therefore, did not suppress 'heat' in these non-fertilizable females but probably enhanced it.

The cyclopentyl enol ether of ENTA, ENTAc-5, proved to be 4 times more potent than ENTA, for inhibition of estrus was produced by 2 µmoles of ENTAc-5 as compared with 8 µmoles of the parent compound; in addition, low dosage levels, *i.e.* 0.5-1.0 µmole, controlled fertility (*i.e.*, as measured by number of deliveries). However, sterility was only temporary because after termination of treatment the majority of females caged with males were fertilized within 10 days (data not tabulated).

Thus, ENTAc-5 appears to be a potent

inhibitor of estrus and conception in the female rat; in addition, it does not interfere with mating, nor have permanent effects on fertility.

Enol etherification by cyclopentylic alcohol, while reducing the anti-estrual and contraceptive activity of MAP, enhances those of ENTA. It is possible that these changes of activity reflect parallel changes in other biological properties; however, the progestational potency of MAPc-5 and of ENTAc-5 as judged by the Clauberg test, and of MAPc-5 as shown by the pregnancy maintenance test in ovariectomized rats[†], appears to remain practically unmodified in comparison with their respective parent compounds. Enol etherification of both MAP and ENTA produces a striking dissociation in the contraceptive and progestional activities of the parent compounds; MAPc-5 is a less active contraceptive and anti-estrual agent than MAP, while ENTAc-5 is 4 times as active as ENTA in this respect. On the other hand, enol etherification of AP neither enhances nor decreases any inhibitory effects on estrus, mating or fertilization within the limits of the experiment.

Summary. Cyclopentyl enol ethers of 17α-acetoxyprogesterone, 6α-methyl-17α-acetoxyprogesterone and 17α-ethynyl-19-nortestosterone acetate were tested for ability to inhibit estrus, mating and fertilization in female rats with the following results: The first compound showed no inhibitory effect; the second blocked the estrous cycle, without interfering with mating and fertilization; the third proved to be a potent antiestrual and contraceptive agent, although it did not disturb sexual receptivity or cause permanent sterility. Thus, enol etherification reduces the antifertility effects of 6α-methyl-17α-acetoxyprogesterone, but strongly enhances those of 17α-ethynyl-19-nortestosterone acetate.

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[†] Unpublished observations (Falconi, G.)

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Comparison of Lipid Metabolism of Chicken Embryo Organs and Cells in Culture.[*] (26828)

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Differences exist in amino acid and carbohydrate metabolism(1,2) between primary cultures and established cell lines. Little is known of the relationship between the metabolism of primary cultures and parent organs. Therefore, a comparison of lipid me-

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tabolism of chicken embryo organs and cells in culture was made. Since at an embryonic stage, cell proliferation is very rapid, conditions may be similar to those that prevail in cell cultures. Incorporation of acetate-1-C^{14} into lipids was studied in cells of chicken embryo *in vivo* and in tissue culture, and also in HeLa and liver (Chang) cells. The isotope concentrations of cholesterol esters, neutral fats, and phospholipids were compared and used as a measure of the metabolism of the lipid fractions.

Materials and methods. The basal medium consisted of Hanks' balanced salt solution with addition of amino acids, vitamins and antibiotics(3). To cultures prepared from chicken embryo 0.1% of lactalbumin hydrolysate (Sheffield Chemicals) was added to stimulate growth. These cells were grown in presence of 2.5% chicken or calf serum (Microbiological Associates, Inc.). HeLa and liver (Chang) cells were cultivated in the same basal medium with addition of 10 or 5% horse serum respectively. Sodium acetate-1-C^{14} (specific activity: 1 millicurie/41 mg (New England Nuclear Corp.) was added to furnish 0.02 mc/100 ml complete medium. For each determination 2-3 bottles of cells were pooled. Each experiment consisted of 3 separate determinations.

Inocula of primary cultures were prepared from organs of 12-day-old chick embryos, as described by Neuman *et al.*(4). Each 250 ml bottle was inoculated with 10 ml of medium containing 4×10^5 cells per ml. After a 3-day period at 37°C the cells were washed with physiological saline, scraped, and washed again.

Inocula of HeLa and liver cells consisted of 10 ml of medium containing about 6×10^4 cells per ml. According to the amount of growth, cells were scraped after 3-4 days, and thereafter treated as described above.

In a manner similar to that described by Marrian *et al.*(5), 0.5 ml acetate-1-C^{14} solution (0.4 mc/10 ml saline) was injected into the fertilized egg air sac under aseptic conditions either on the 9th or 11th day of incubation. On the 12th day, the embryo organs were isolated, washed and extracted for lipids. Preliminary experiments had shown that 5 mg sodium acetate per egg could be tolerated without apparent damage to the embryo. Each determination was done by pooling organs from at least 3 embryos. Each experiment consisted of 3 separate determinations.

Lipids of washed cells or organs were extracted and washed several times as described by Folch, *et al.*(6). Lipid fractions were separated on silicic acid columns(7) into: 1. cholesterol esters; 2. neutral fats, free fatty acids, and cholesterol; and 3. phospholipids. The neutral fat fraction was rechromatographed on short alumina columns(8). Free cholesterol present in fraction 2 was evaluated, using the digitonin method(9). C^{14} activities of various fractions were measured as ultrathin specimens with a thin-window Geiger tube. Serum fatty acids were analyzed using a gas chromatographic method (10). Nitrogen was determined with Nessler reagent(11), serum cholesterol according to the method of Abell *et al.*(12), and total serum fatty acids by the Albrink method(13).

Results. When fertilized eggs were injected with acetate-1-C^{14} and incubated either 24 or 72 hours, the relative C^{14} incorporation in embryonic tissues was greatest in phospholipids ($>75\%$), and less in neutral fats and cholesterol esters (Table I). Total incorporation was maximal in stomach, less in thigh, and still less in heart, and was higher after the shorter incubation period. Actual C^{14} content of the cholesterol ester fractions of the 3 organs was fairly constant, but based on relative distribution within the lipids of a given tissue the cholesterol ester fraction of the heart was highest and that of stomach lowest.

In experiments employing cell cultures from chick embryo heart, thigh, and stomach more than 70% of lipid radioactivity was present in the neutral fat fraction, with most of the remainder in the phospholipids, and a small percentage in the cholesterol ester fraction (Table I). Total radioactivity/mg protein was considerably higher in lipids of thigh and stomach grown in presence of calf serum, than when chicken serum was present. The relative percentage of C^{14} in the cholesterol ester fraction was significantly higher for all

TABLE I. Incorporation of Acetate-1-C^{14} into Cholesterol Esters (CE), Neutral Fats (NF), and Phospholipids (PL) in Chicken Embryo Organs and Cultures Derived from Them.

		Relative C^{14} distribution			
		Organs		Cultures	
Organ lipid fractions		24 hr*	72 hr*	72 hr* Chicken serum	72 hr* Calf serum
		%			
Heart	CE	3.1 ± .06†	4.3 ± .7	2.8‡ (2.0- 3.6)	.8‡ (.8- .8)
	NF	20.5 ± 1.9	20.3 ± .7	81.9 (80.5-83.2)	80.6 (75.5-85.7)
	PL	76.4 ± 1.9	75.4 ± .3	15.4 (13.2-17.5)	18.6 (13.5-23.7)
Total cpm/mg protein		346	155	14,487	13,090
Thigh	CE	1.7 ± .35	2.8 ± .47	1.9 ± .20	.9 ± .0
	NF	13.3 ± .5	16.8 ± .4	75.9 ± 3.9	65.0 ± 1.7
	PL	84.9 ± .7	80.4 ± .2	22.1 ± 4.1	33.9 ± 1.7
Total cpm/mg protein		995	344	11,855	19,383
Stomach	CE	.9 ± .07	1.1 ± .17	4.8 ± .18	2.2 ± .46
	NF	15.8 ± 1.4	17.0 ± .6	81.3 ± .3	78.3 ± 2.6
	PL	83.2 ± 1.4	81.9 ± .7	13.9 ± .9	19.5 ± 2.6
Total cpm/mg protein		1,352	572	11,882	18,580

* Period of incubation with acetate-1-C^{14}.
† Mean ± stand. error.
‡ Two determinations. Two pools of 3 bottles each were used.

3 tissues grown in chicken serum as compared with calf serum. As judged by a *T* test(14) the significance of these differences between the means for thigh and muscle was $P<0.01$. Radioactivity due to free cholesterol and unesterified fatty acids in the neutral fat fractions was negligible.

The radiocarbon content of the lipids of 2 established cell lines, HeLa and liver (Chang), grown in presence of acetate-1-C^{14} was highest in phospholipids, lower in neutral fat, and very low in the cholesterol ester fractions (Table II).

Discussion. Lipid C^{14} of chicken embryo organs from fertilized eggs injected with acetate-1-C^{14} was present predominantly in phospholipids. These results resembled those obtained with rats fed acetate-1-C^{14} for 24 or 72 hours in which most lipid C^{14} was recovered in phospholipids(15). In cells cultured from chicken embryo organs, however, neutral lipids contained most of the lipid C^{14}. Radiocarbon distribution in lipids of HeLa and liver (Chang) cells was similar to that of conjunctival (Chang) cells(16). Marks *et al.*(17) found radioacetate mainly incorporated into neutral lipids of normal leukocytes, but equally into neutral fats and phospholipids of leukemic cells. Therefore, of cells so far studied *in vitro* only those cells which were non-malignant or incapable of unlimited rapid and autonomous multiplication, incorporated most of the radioacetate into neutral fat. Further, from the standpoint of acetate incorporation, such cells bore the least metabolic resemblance to metabolism *in vivo*.

TABLE II. Isotope Content of HeLa and Liver Cell Lipids.

	% of C^{14} concentrations	
Fraction	HeLa*	Liver*
Cholesterol esters	.4 ± .06†	.5 ± .15
Neutral fats	42.9 ± 1.9	43.0 ± .9
Phospholipids	56.7 ± 1.8	56.5 ± 1.6
Total cpm/mg protein	27,507	23,417

* HeLa cells were grown for 3 days; liver cells for 4 days.
† Means and stand. errors.

Exogenous lipids gain access to tissue culture cells(18,19); however, the influence of kind of serum on relative incorporation of acetate into cholesterol esters (Table I) cannot be explained readily on the basis of degree of unsaturation, fatty acid composition, or total fatty acid and cholesterol content (Table III). This is in agreement with

TABLE III. Fatty Acid and Cholesterol Composition of Chicken and Calf Serum.*

Fatty acid		% composition	
No. of carbons	Degree of saturation	Chicken	Calf
C_{12}	saturated	trace	.5
C_{14}	"	.4	2.5
C_{15}	"	.1	.9
C_{16}	"	12.1	11.9
C_{16}	monoenoic	2.0	3.6
C_{18}	saturated	19.5	14.2
C_{18}	monoenoic	32.0	24.8
C_{18}	dienoic	25.6	24.7
C_{18}	trienoic	.4	8.8
C_{20}	tetraenoic	5.9	3.8
Unsaturated fatty acids (%)		65.9	65.7
Saturated " " (%)		32.1	30.0
Total cholesterol (mg %)		138.0	152.0
" fatty acids (meq/l)		18.3	24.4

* Based on the gas chromatography(14) of normal and hydrogenated methyl esters using 20% Craig polyester succinate on 60-80 mesh Chromosorb W (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) at 180°C and argon flow rate of 60 ml/min. A Research Specialties Co. instrument equipped with a strontium ionization detector was used. Small amounts of other acids were present, but comprised less than 5% of the total and were omitted from the results.

Bailey(20) who showed cholesterol uptake of mouse lymphoblasts (MB III) was influenced by the kind of serum used, but not by the cholesterol or total fatty acid content of the serum itself. He concluded that the relationship between serum cholesterol and the binding power of the serum proteins may be the most important factor. No influence of exogenous stearic or linoleic acids was noted. Further studies are required to elucidate fully the mechanism(s) of the action of serum. Primary cultures, such as those of chicken embryos, as well as established cell lines, should be of value in studies of cholesterol ester and lipid metabolism as affected by various drugs and nutrients.

Summary. A study was made of the relative incorporation of acetate-1-C^{14} into lipids of chicken embryo tissues *in ovo* and in tissue culture. Embryonic heart, thigh, and stomach incorporated C^{14} mainly into phospholipids *in ovo,* and into neutral fat *in vitro.* Relative C^{14} content of the cholesterol esters was higher when the cells were grown in medium containing calf serum as compared with chicken serum, an effect not correlated with serum fatty acid composition or fatty acid and cholesterol content. Acetate incorporation by established cell lines (HeLa and Chang liver) resembled more closely that of chick embryo organs *in ovo* rather than that of chick embryo tissues *in vitro.*

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Effects of Avian and Rat Pituitary Extracts on Tibial Growth and Blood Composition.* (26829)

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Data are available which indicate that differences exist in molecular structure as well as in physiological effects of growth hormone preparations obtained from various species (1). Thus, employing the rat tibia test, hypophyseal extracts from human, whale, monkey and beef sources were reported to be nearly equal in potency, yet differ in their ability to promote gains in body weight(2). Solomon and Greep(3) compared the growth promoting potential of pituitary extracts from 9 different vertebrate forms and concluded from rat epiphyseal cartilage data that the hypophysectomized recipients were more sensitive to amphibian and mammalian preparations than they were to extracts prepared from fish. Also, these authors reported that the hypophysectomized rat was insensitive to avian pituitary extracts; however, the results presented were for one dose level which was assayed in only 4 hypophysectomized animals.

Additional information on the growth hormone content of chicken pituitaries seemed desirable, especially in light of the multiple differences with respect to protein anabolic and carbohydrate metabolism known to exist between aves and mammalia, differences which are readily influenced by administration of growth hormone(4). Whether the avian growth hormone molecule differs structurally from that of other species, or whether avian metabolism is sufficiently different from that of mammals to render the bird resistant to mammalian growth hormone, is not clear. However, adult chickens have been reported to be very resistant to the protein anabolic as well as to the carbohydrate influence of growth hormone prepared from mammalian sources(5,6).

The purpose of this study, therefore, was to investigate comparative potency of avian, rat and purified beef growth hormone as assayed in the immature hypophysectomized female rat. The parameters chosen for assaying these preparations were the tibia cartilage width, blood urea, oxyhemoglobin, hematocrit and whole blood specific gravity (WBSG).

Methods. Chicken heads from 9 to 12-week-old White Rock birds of both sexes (equally divided) were obtained immediately after death (Broadway Poultry Co., Boston, Mass.), the anterior pituitary separated rapidly from the posterior lobe, weighed on a Roller-Smith torsion balance, and pulverized manually in a Potter homogenizer containing a known amount of saline. These crude extracts were then made alkaline, butanol added as a preservative, and stored at 0-4°C. Each extract was made fresh 48 hours prior to first injection. Rat pituitaries were obtained from young adult Sprague-Dawley descendents of both sexes (equally divided) and were extracted in the same manner. Purified beef growth hormone (NIH-GBH-2) was donated by the Endocrinology Study Section.

Pituitary preparations were injected subcutaneously once each day (in equally divided doses over a 4-day period) into female rats hypophysectomized at 26 days of age (Charles River Breeding Laboratories) and allowed to recover an additional 17 days prior to use. Control rats received 0.25 ml of 0.9% saline solution per injection. Eight hours after last injection all recipients were fasted 16 hours, after which a terminal blood sample was drawn from the vena cava, and the right tibia excised and placed in 10% formol to be stained with silver nitrate according to the method of Greenspan *et al.*(7). Microhematocrits, oxyhemoglobin(8) and specific gravity(9) determinations were carried out on fresh whole blood; serum urea was measured by the method of Rosenthal(10). Completeness of hypophysectomy was judged at

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* This study was supported by NSF Grant.

TABLE I. Assay of Avian, Rat and Purified Beef Growth Hormones.

Donors		Recipients						
No. of ant. pit.	Ant. pit. wt, mg	No. of rats	Body wt gain, g	Tibia width, μ	Blood urea, mg %	Oxyhemoglobin, g %	Hematocrit, %	Blood specfic grav.
Total dose/4 days				Avian pituitary extract				
NaCl control*	—	15	0	153 ± 2†	62.8 ± 1.1	16.5 ±.27	36.2 ± .9	1.048 ±.0006
½ ant. pit.	3.3	9	4	162 ± 2	55.4 ± 1.3	15.7 ±.49	32.9 ±1.0	1.044 ±.0008
1 *idem*	6.6	10	4	191 ± 6	55.6 ± .9	15.6 ±.33	32.5 ± .9	1.045 ±.0009
2 "	13.2	11	1	236 ± 5	52.9 ± 1.3	14.3 ±.38	31.3 ± .8	1.044 ±.0005
4 "	26.4	8	1	285 ±15	49.2 ± .6	15.1 ±.35	31.5 ±1.1	1.045 ±.0011
				Rat pituitary extract				
¼ ant. pit.	1.6	8	3	242 ± 4	53.3 ± 1.0	—	—	—
½ *idem*	3.2	8	4	293 ± 6	54.1 ± 1.2	16.1 ±.40	34.2 ± .5	1.046 ±.0005
1 "	6.5	8	6	312 ± 9	49.4 ± 1.3	15.5 ±.25	33.7 ± .7	1.045 ±.0005
				Purified beef growth hormone				
NaCl control	—	11	.3	156 ± 4	66.0 ± 2.5	17.3 ±.20	36.2 ± .5	1.049 ±.0004
100 μg	—	8	5	207 ± 7	56.1 ± 1.6	16.9 ±.26	34.0 ± .6	1.047 ±.0005
200 "	—	6	7	225 ± 9	58.1 ± 1.7	16.4 ±.33	31.9 ± .9	1.047 ±.0006
400 "	—	7	8	303 ±15	45.5 ± 2.1	16.0 ±.31	31.9 ± .5	1.046 ±.0004
1600 "	—	8	16	407 ±13	56.3 ± 2.7	15.9 ±.48	32.0 ±1.1	1.046 ±.0009

* Control rats received 0.25 ml NaCl daily.

† All values are means ± stand. error.

autopsy by adrenal weights and examination of the sella turcica.

Results. Table I compares the results of injecting graded doses of avian and rat anterior pituitary tissue with those of a highly purified beef preparation. Equal growth-promoting properties were evident when tibias of rats receiving 13.2 mg avian hypophyseal tissue were compared with those of rats receiving 1.6 mg rat pituitary extract. By bioassay standards of significance(7,11) a significant increase in cartilagenous width required 6.6 mg avian hypophyseal extract ($p<.01$) whereas the minimum rat pituitary dose employed (1.6 mg) evoked a highly significant increase in tibial size ($p<.001$).

While the lowest dose of avian pituitary extract did not effectively increase tibial cartilage width, it decreased significantly urea ($p<.01$), hematocrit ($p<.05$), and WBSG levels ($p<.01$). Oxyhemoglobin was depressed significantly only when the equivalent of 2 or more avian pituitaries were injected ($p<.05$).

Discussion. The results of this study confirm and extend an earlier report on avian hypophyseal growth substance(s) as evidenced by the epiphyseal cartilage assay test (3), and present observations as to certain hematological alterations induced by these adenohypophyseal extracts. Comparing tibial cartilage widths, the chicken anterior pituitary apparently contains one-eighth of the growth-stimulating potential found in equal amounts of the rat adenohypophysis. It is possible that the hypophysectomized rat is considerably more resistant (8 times?) to avian hypophyseal extracts; however, it would be necessary to conduct assays in immature hypophysectomized chicks to distinguish a decrease in hypophyseal hormone content from an alteration in recipient resistance.

The hematological response to avian pituitary extracts in absence of tibial growth is of interest, for as such, these indices may aid in evaluation of such avian preparations. The depression in oxyhemoglobin, hematocrit and WBSG levels induced by the purified beef preparation are in accord with observations using similar preparations in normal and diabetic dogs(12). However, considerable variability occurred in all blood parameters at the highest dose level of purified growth hormone employed. From the blood data in Table I it would appear that the minimal effective dose (as judged by tibial enlargement) would nonetheless evoke a marked depression in urea levels.[†] The qualitative similarity in tibial and hematological responses to both crude hypophyseal extracts and purified preparations would implicate the growth hormone molecule in effecting these alterations. However, since assay of other hypophyseal hormones contained in these crude extracts was not carried out their possible influence on the parameters chosen for study cannot be assessed here.

[†] With saline control tibial widths ranging 145-160 μ a significant response is generally considered in excess of 190 μ(7, 11).

The failure to observe a linear response in body weight gain may be due in part to the short fasting time employed (and therefore variable intestinal contents), or may indicate a sensitivity of the hypophysectomized rat to the crude hetero-specie material injected.

Summary. Chicken and rat hypophyseal extracts were compared with a purified beef growth hormone preparation in the hypophysectomized, immature female rat using the tibial cartilage assay and hematological alterations. The avian adenohypophysis contains ⅛th of the growth-promoting material found in an equivalent amount of rat hypophyseal extract. Significant depression in hematocrit and WBSG levels occurs at doses of avian extracts which are unable to promote epiphyseal cartilage growth.

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Extraction of a Glucose Uptake Stimulator from Tumors.* (26830)

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The presence of a glucose uptake stimulator in a retroperitoneal fibrosarcoma has recently been reported from this laboratory(1). This tumor had been removed from a patient exhibiting repeated episodes of spontaneous hypoglycemia associated with increased plasma insulin-like activity. Following removal of the tumor, the patient became euglycemic and had plasma insulin-like activity indistinguishable from normal, which indicated that the tumor was the source of a glucose uptake stimulator(2). These observations suggested the possibility that a glucose uptake stimulator might be found in other neoplastic tissues even in the absence of hypoglycemic symptoms in the tumor-bearing patients. This paper reports the results of assays for a glucose uptake stimulator in various malignant tumors.

Methods. Tumors obtained from the operating room within minutes of removal from the patients were carefully dissected away from surrounding tissues, cut into pieces 3-4 cm thick, and stored at –20°C until use. In most cases it was possible to obtain tissues showing no histological evidence of neoplastic changes from the areas surrounding the tumor. Fractions were prepared from these tissues in the same manner as that used for

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the tumors and will be referred to as "normal" tissue fractions.

The fractions were prepared as follows: All manipulations were carried out at 3-5°C. Five g of tissue were homogenized in Krebs Ringer Bicarbonate buffer containing 2.5 mg/ml glucose(3). The homogenate was centrifuged and the supernatant brought to a 22% ethanol concentration. The precipitate was collected by centrifugation and was saved for assay. The supernatant was brought to a final ethanol concentration of 50% and the resultant precipitate was collected by centrifugation and saved for assay. For assay the precipitates were all suspended in buffer containing 2.5 mg/ml glucose by use of a Potter-Elvehjem homogenizer.

Assessment of the glucose-uptake stimulating activity (hereafter referred to as the stimulator) was made on the basis of an increase in glucose uptake by epididymal fat pads in the presence of a particular fraction over that in buffer alone. A control group was included in every incubation. No attempt has been made to quantitate the amount of stimulator in terms of units of insulin activity for the reasons given previously(1). Assays were carried out by measuring glucose uptake of intact fat pads from rats weighing 150-200 g. Glucose concentration was measured by the method of Somogyi(4). Details of the procedure have been reported(1).

Results. The data shown in Table I are representative assays of malignant tissues removed from different patients. Included are 3 different adenocarcinomas of the breast, 3 adenocarcinomas of the colon, one adenocarcinoma of the kidney, and one liposarcoma. "Normal" tissues including skin, colon, and kidney dissected from around the tumors were fractionated and assayed in a manner identical to that used on the tumors.

All 3 of the adenocarcinomas of the breast (Groups 1-3) yielded precipitates at both the 22%† and the 50% ethanol concentration which caused a significant increase in glucose uptake. The same was true of the adenocarcinoma of the kidney (Group 4) and 2 of the 3 adenocarcinomas of the colon (Groups 6 and 7). One colon tumor was without significant activity in either the 22% or 50% precipitate (Group 8). Most of the stimulating activity in the liposarcoma was concentrated in the 22% precipitate since addition of ethanol from 22 to 50% gave a precipitate with no significant activity.

Of the normal tissues assayed, neither the 22% nor the 50% precipitates of pectoral muscle nor skin stimulated glucose uptake significantly (Groups 9, 10, 13 and 14). In the case of the colon one specimen did not show a stimulating activity (Group 12) while another specimen showed stimulation in the 22% ethanol fraction (Group 11). Comparison of the activities in the normal colon tissue (Group 11) with the tumor tissue (Group 6), from this patient would indicate that the tumor had appreciably more stimulator than the normal tissue in both the 22% and the 50% ethanol precipitates. Kidney (Group 15) had as much activity as the kidney adenocarcinoma (Group 4) in the 22% fraction; however, increasing the ethanol concentration to 50% did not result in an increase in activity in the normal in contrast to the tumor.

Discussion. It is clear that all but one of the tumor fractions stimulated glucose uptake of the epididymal fat pad significantly. Corresponding fractions of normal tissue either had no stimulator or much less stimulator than did the tumors. It is immediately apparent that the stimulator has not been concentrated in a single fraction by the technics employed here. Previous experience with a retroperitoneal fibrosarcoma would have suggested that the stimulator could be concentrated by forming a 22-50% ethanol precipitate or a zinc acetate precipitate. This observation did not prove to be true except in isolated instances (Groups 2, 4 and 5). Also in contrast to our experience with the fibrosarcoma, it was not possible to remove a glucose uptake inhibitor from the fractions by dialysis. Continuing attempts to concentrate the stimulator by other procedures have not been markedly successful.

Most recent efforts to demonstrate a glu-

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† The terms 22% and 50% ethanol precipitates refer to precipitates formed by addition of ethanol from 0-22% and 22% - 50%.

TABLE I. Glucose Uptake of Rat Epididymal Fat Pads Incubated in Ethanol Precipitates of Tumors and ''Normal'' Tissues, mg/g Fat Pad/3 Hours.

Group	Tissue	Control	22% ppt.	p*	Change‡	50% ppt.	p*	Change‡
			Neoplastic tissues					
1.	Breast Ca (P)	2.66 ± .28 (6)†	4.56 ± .47 (6)†	<.01	+1.90	5.70 ± .91 (6)†	<.02	+3.04
2.	" " (W)	2.42 ± .26 (5)	3.94 ± .42 (5)	<.02	+1.52	4.61 ± .17 (5)	<.001	+2.19
3.	" " (F)	.98 ± .08 (5)	2.81 ± .24 (5)	<.001	+1.83	2.05 ± .42 (5)	<.05	+1.07
4.	Kidney Ca (I.W.)	2.06 ± .17 (5)	4.19 ± .38 (5)	<.001	+2.13	6.33 ± .26 (5)	<.001	+4.27
5.	Liposarcoma (S)	2.65 ± .22 (5)	6.03 ± .81 (5)	<.01	+3.38	3.55 ± .63 (5)	n.s.	+ .90
6.	Colon Ca (Ba)	2.15 ± .33 (5)	5.11 ± .50 (5)	<.01	+2.96	4.10 ± .68 (5)	<.05	+1.95
7.	" " (Bu)	1.93 ± .26 (5)	3.43 ± .52 (5)	<.05	+1.50	3.65 ± .57 (5)	<.01	+1.72
8.	" " (C)	1.31 ± .31 (5)	2.25 ± .36 (5)	n.s.	+ .94	1.48 ± .04 (5)	n.s.	+ .17
			''Normal'' tissues					
9.	Pectoral mus. (W)	2.42 ± .26 (5)	2.88 ± .61 (5)	n.s.	+ .46	3.02 ± .24 (5)	n.s.	+ .60
10.	" " (H)	1.01 ± .17 (5)	1.70 ± .28 (5)	"	+ .69	1.46 ± .17 (5)	"	+ .45
11.	Normal colon (Ba)	2.15 ± .33 (5)	3.98 ± .46 (5)	<.02	+1.83	3.15 ± .31 (5)	"	+1.00
12.	" " (C)	2.36 ± .31 (5)	2.14 ± .53 (5)	n.s.	− .22	2.58 ± .65 (5)	"	+ .22
13.	Skin (H)	1.89 ± .10 (5)	2.40 ± .85 (5)	"	+ .51	2.00 ± .50 (5)	"	+ .11
14.	" (W)	2.05 ± .37 (5)	2.69 ± .56 (5)	"	+ .64	2.42 ± .38 (5)	"	+ .37
15.	Kidney (I.W.)	2.06 ± .17 (5)	4.23 ± .55 (5)	<.01	+2.17	3.45 ± .41 (5)	<.02	+1.39

* p compares significance of the difference of means of experimental group to buffer control group. p is derived from t where

$$t = \frac{m_1 - m_2}{\sqrt{\left(\frac{\Sigma d_1^2 + \Sigma d_2^2}{n_1 + n_2 - 2}\right)(1/n_1 + 1/n_2)}}$$

n.s. = not significant

† Mean ± stand. error of mean. No. in parentheses is No. of fat pads in each group.

‡ Change is the difference of experimental group from control group.

cose uptake stimulator in tumor tissue have been directed toward a study of those tumors associated with spontaneuos hypoglycemia. Of the several attempts (see 5 for review) only 2 have been successful(2,6). Shortly after the discovery of insulin, however, several investigators demonstrated a material in tumor extracts not associated with hypoglycemia capable of modifying the carbohydrate metabolism of normal tissues. Waterman (7) showed that extracts of human carcinoma, mouse carcinoma, and sarcoma increased glycolysis of normal kidney slices and postulated the material acted at the first steps involved in glucose utilization. The tumor extracts had more of the glycolysis activator than did normal tissues. Brooks and Jowett(8) using a somewhat different procedure, failed to confirm this observation. Others demonstrated insulin-like activity in various malignant tissues(9,10) as well as in normal tissues(11). Most recently, Silverstein *et al.*(12) have demonstrated a hypoglycemic factor in leukemic tumors extractable with acetone but not with the usual insulin extraction procedure.

The data reported here and the observations referred to above suggest that a material capable of stimulating glucose uptake *in vitro* is associated with at least several different malignant tissues and perhaps some normal tissues. With the extraction method employed here there is more stimulator present in tumors than in surrounding, presumably, non-neoplastic tissue.

A discussion of the identity of the glucose uptake stimulator is not now profitable for two reasons. The stimulator has not been adequately concentrated and purified. Secondly, it has been demonstrated that glucose uptake of epididymal fat pads is stimulated not only by insulin(13), but also by growth hormone(14,15), ACTH(15,16), and epinephrine(16,17). Because of these observations, and in view of the differences in activity in comparable fractions of the different tumors reported here, it is entirely possible that more than one factor is responsible for stimulation of glucose uptake.

Summary. A material capable of stimulating glucose uptake of rat epididymal fat pads *in vitro* has been extracted and concentrated by ethanol precipitation from several neoplastic tissues. Most of the normal tissues assayed did not have significant amounts of the glucose uptake stimulator. In the 2 cases where a stimulator was found in normal tissues, it was present in lower concentrations than in the corresponding tumors.

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Volume Receptors and Post-prandial Diuresis in the Seal (*Phoca vitulina* L.)* (26831)

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Acute changes in urine flow in the seal have interested physiologists since Hiatt reported the post-prandial diuresis which occurs 2 to 4 hours after feeding. This diuresis is accompanied by hypertonic urine and an increase in glomerular filtration rate(1). Schmidt-Nielsen and co-workers confirmed these findings(2).

Attempts to reproduce this pattern of change with diuresis in the seal using various types of osmotic diuresis or hydration failed unless the seal had been fed(1,2). This unexplained form of diuresis stimulated this inquiry into the possible existence of a volume receptor in the seal.

The results of this study show that plasma volume expansion produces the pattern of diuresis seen post-prandially and suggest the presence of a volume receptor in the seal.

Methods. Four young female harbor seals, weighing 20 to 25 kg, were studied at the Mount Desert Island Biological Laboratory after a short period of adjustive training. They were maintained on a diet of fresh herring, lived in wooden enclosures floated in the bay and appeared in good health throughout the studies. Because seals in captivity frequently develop subcutaneous infections, Bicillin® was administered to each seal every 2 weeks. No anesthesia was used except local anesthesia for venipunctures.

The seals were studied in the fasting state comfortably laced into a canvas sling suspended by ropes. A gentle swinging motion of the sling and soft music were used to calm the seal. The seal was periodically wetted with water to prevent dehydration.

Three types of experiments were conducted: continuous negative pressure breathing, expansion of plasma volume, and water infusion studies.

The continuous negative pressure breathing studies were performed using a plastic box with a hole to fit the neck of the seal snugly when its head was inserted into the box. Suction was applied to the box and maintained at minus 20 cm water using a water trap. Soda lime containers were placed in the box to prevent carbon dioxide accumulation. Plasma volume expansion was produced using 400 ml 1% gelatin in saline in each seal, and 400 ml fresh seal plasma in one seal, administered intravenously over a 50 minute period.† On a different day each seal was given by intravenous route 400 ml 5% dextrose in water over a 50 minute interval. Studies were preceded by a 2 to 4 hour observation period to assure a stable state and urine flow.

Intravenous infusions contained creatinine and were administered through an intravenous cannula in a fore-flipper using a constant infusion pump. The infusion rate during control observation periods was 0.6 ml/min which is less than the seal's normal fluid intake(2,3). Creatinine concentration in the test infusion fluids (gelatin, plasma, and water) was adjusted to obtain the same minute creatinine administration given during the control observation periods.

Urines were collected at 20 to 30 minute intervals *via* an inlying catheter, and heparinized blood samples were obtained at 30 to 60 minute intervals. Urine and plasma samples were analyzed for sodium and potassium content using a flame photometer, creatinine concentration by Hare's method without Lloyd's reagent(4), and osmolality using a

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* Supported by grants from Alabama Heart Assn. and U.S.P.H.S.

† Dextran was not used because the seal excretes it in a quantity sufficient to produce a viscous urine.

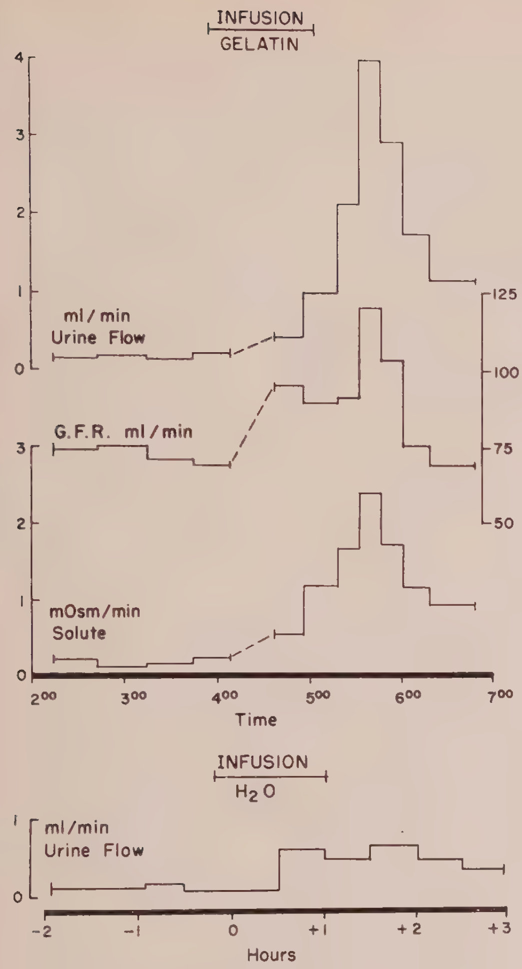

FIG. 1. Effect of infusion of 400 ml 1% gelatin in 0.9% NaCl in a harbor seal (above). Change in urine flow in response to the same volume of water infused in the same seal (below).

Fiske osmometer. Urines were checked for gelatin during the experiment using silver nitrate, and total urine protein determinations were performed later by Nesslerization.

Results. Continuous negative pressure breathing for periods up to one hour did not cause an increase in rate of urine flow in any of the 4 seals. Infusion of 5% dextrose in water did cause increase in rate of urine flow, but this increase was small in comparison to the changes found with expansion of plasma volume (Fig. 1). No change in glomerular filtration rate was caused by water administration.

Expansion of plasma volume by gelatin infusion or by plasma infusion provided a pronounced increase in rate of urine flow (Fig. 1). With this diuresis, there occurred an increase in glomerular filtration rate as measured by exogenous creatinine clearance, concentrated urines with osmolal U/P greater than 2 at peak of diuresis, and a solute diuresis without change in plasma osmolality. No gelatin was found in the urine during diuresis in response to gelatin infusion.

Discussion. Man's response to an increase in plasma volume is a water diuresis without change in glomerular filtration rate(5), but the response of the seal to the same stimulus is a solute diuresis with hypertonic urine and an increase in glomerular filtration rate. This diuresis indicates the presence of a volume receptor in the seal but implies that it is of a different mechanism than that of man.

This pattern of diuresis in response to increase in plasma volume in the seal is like that seen in the post-prandial diuresis. It would therefore appear likely that the post-prandial diuresis is mediated *via* a volume receptor in response to increase in plasma or blood volume after feeding.

A seal swimming with its head above water breathes against a relative negative pressure comparable to a man breathing while immersed in water to his neck. In such a situation man experiences a water diuresis(6). Continuous negative pressure breathing, another situation where pressure against the exterior of the body is greater than the pressure respired against, causes a water diuresis in dog(7) and man(8,9). These findings have been interpreted as evidence for intrathoracic volume receptors which initiate changes in rate of urine flow with changes in thoracic blood volume.

The finding that the seal will tolerate prolonged periods of continuous negative pressure breathing without change in urine flow indicates that the animal does not have atrial stretch receptors or that he has some adaptive mechanism to prevent displacement of blood into the thorax. The seal's inferior vena cava valve may be such an adaptive mechanism.

Summary. The effect of continuous negative pressure breathing and of plasma volume expansion was studied in young harbor seals. Negative pressure breathing did not increase rate of urine flow. Expansion of plasma volume with seal plasma or with 1% gelatin in saline caused a solute diuresis with increase in glomerular filtration rate similar to the changes seen in the post-prandial diuresis of the seal. It is suggested that the post-prandial diuresis of the seal is mediated *via* a volume receptor.

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Adrenocortical Response to Stress in Rats with Lesions in Hippocampus and Amygdala.* (26832)

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A continually mounting volume of research, summarized regularly(1-3), has explored mechanisms of neural regulation of ACTH secretion. The most intense interest is centered about isolation and characterization of the ACTH-regulating neurohumor(4-7) and identification of the hypothalamic area(s) in the region of the median eminence which represent the terminal link(s) between the central nervous system and the adenohypophysis(8-11). More recently attention has been directed also to other regions of the brain which may have direct connections with the final hypothalamic effector sites or which may influence indirectly the amount of ACTH neurohumor produced there. Among these areas are the cerebral cortex(12,13), limbic system(14,15), brain stem and reticular formation(12,16-18). The present report describes alterations in basal levels of plasma free corticoids (PFC) and in the pattern of PFC response to stress occurring in rats with lesions of hippocampus or amygdala.

Materials and methods. Bilateral electrolytic lesions (3 ma, 30 sec) were placed by stereotaxic means in hippocampus, amygdala and habenular complex of adult male rats. The insulated stainless steel needle used for lesioning was bared approximately 1 mm at its tip for placement of lesions in amygdala and habenula; for hippocampus, the needle tip was bared 1.5-2 mm. Using a stereotaxic instrument with reference planes adjusted to the atlas of de Groot(19), the anterior vertical and lateral coordinates used for lesioning amygdala, hippocampus and habenula were (+5.4, –2.8, ± 4.5), (+3.0, –2.5, ± 5.0) and (+3.4, +0.8, ±0.5) respectively. Two months after lesioning, I^{131} uptake was measured by counting the external neck activity 18-24 hrs after intraperitoneal administration of 5 μc carrier-free NaI^{131}; a region of the hind limb was counted as a measure of the non-thyroidal neck activity. After one additional month, peripheral PFC levels were determined. Basal PFC levels and PFC response to 30 min and 4 hrs of continuous physical immobilization (paws bound with adhesive tape) were examined in both control and lesioned animals. Animals were sacrificed by decapitation, blood collected in heparinized beakers and PFC determined fluorometrically according to Guillemin *et al.*

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* Supported by U.S.P.H.S. and N.S.F. grants.

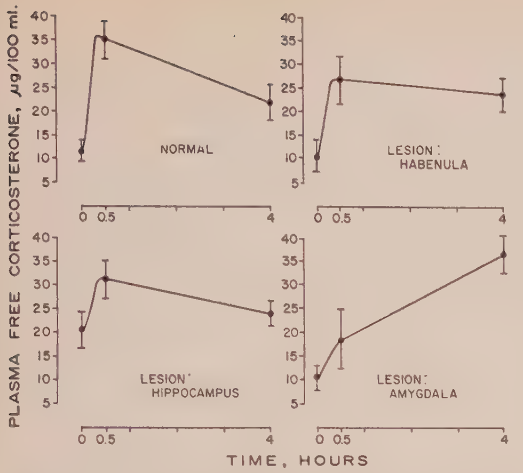

FIG. 1. Plasma free corticosterone response to immobilization stress in rats with lesions in habenula, hippocampus, amygdala.

(20). Brains of all lesioned animals were fixed in 10% formalin and position of lesions determined by reconstruction from 75 μ frozen sections. Sections of thyroid, adrenal and testis of control and lesioned animals were prepared also for histological examination.

Results. Thyroid and testis. Two months after lesioning, thyroid I^{131} uptake of all experimental groups was not significantly different from that of control animals (Table I); also, thyroid weight and histological appearance were not changed when examined at time of sacrifice 3 months after lesioning. No impairment of pituitary gonadotropic activity was evident as judged by testicular weight or histology.

Adrenal. Resting PFC levels in normal rats were 11.8 ± 2.1 μg/100 ml; immobilization stress evoked a 200% increase (35.1 μg/100 ml) in PFC at 30 min which declined to 22.4 μg/100 ml after 4 hrs of continuous immobilization (Table I; Fig. 1). PFC values of similar magnitude have been reported to occur in the rat after such stressors as ether anesthesia, painful stimuli(21), formalin injection(22) and electroshock(23). In animals with lesions in the habenular complex, the mean PFC response after 30 min of stress was not as great as in normal animals, nor was the decline after 4 hrs of stress to the same level as normal; at both time intervals, however, PFC values were not statistically different from normal. After electrolytic destruction of relatively large areas of hippocampus, resting PFC levels of 20.6 ± 3.8 μg/100 ml were significantly higher than normal; in response to stress of immobilization, PFC levels after 30 min and 4 hrs were in the same range as in non-lesioned animals. Bilateral destruction in the amygdaloid nuclei did not alter resting PFC levels (Table I); the pattern of response to immobilization stress, however, was notably different from normal or hippocampal-lesioned animals. Only moderately elevated and not statistically different PFC levels were observed after 30 min and this rose to 37.0 ± 3.8 μg/100 ml after 4 hrs (Fig. 1).

Three months after placement of these relatively large lesions in hippocampus and amygdala, reconstruction of the exact extent of the lesions was difficult because of collapse of the nervous tissue around the lesion site, involvement and destruction of the lateral ventricular walls and distortion of the normal contour of the temporal lobe. In Fig. 2 the area common to the lesions placed in hippocampus and amygdala is presented as reconstructed from the brains of 6 animals of each

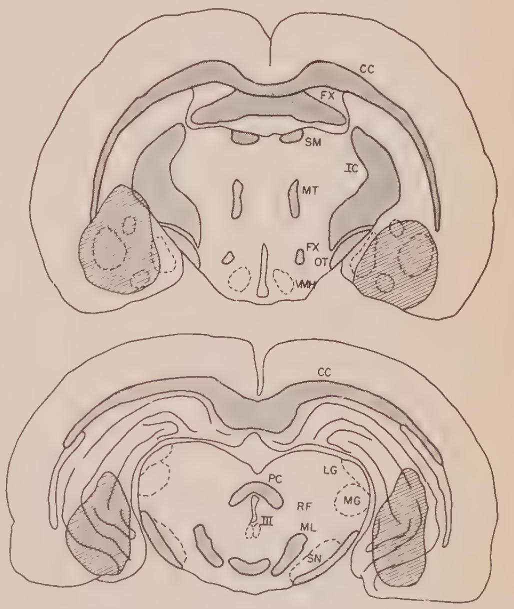

FIG. 2. Localization of lesions in amygdala (upper section) and hippocampus (lower section) which produced alterations in basal PFC levels and in PFC response to immobilization stress.

TABLE I. Endocrine Function and Response to Stress in Rats with Lesions in the Limbic System.

Group	No.	Testis, g	Thyroid, mg	I^{131} uptake, % A.D.	Adrenal, mg	P.F.C., μg/100 ml
Non-lesioned						
a. Non-stressed	9	1.60 ± .71	16.7 ± 1.9	9.18 ± .36	23.9 ± 3.1	11.8 ± 2.1
b. Stressed, .5 hr	12	1.70 ± .14	15.9 ± 2.0		25.2 ± 2.3	35.1 ± 4.0
c. " , 4 "	12	1.66 ± .10	15.9 ± 2.0		23.8 ± .7	22.4 ± 3.8
Lesioned: Hippocampus						
a. Non-stressed	10	1.68 ± .08	17.3 ± 3.2	10.09 ± 1.27	21.2 ± 2.3	20.6 ± 3.8
b. Stressed, .5 hr	7	1.74 ± .10	14.8 ± 2.4		24.6 ± 2.0	31.2 ± 4.1
c. " , 4 "	9	1.65 ± .09	15.8 ± 1.6		24.0 ± 2.5	24.2 ± 2.5
Lesioned: Amygdala						
a. Non-stressed	10	1.67 ± .17	16.5 ± 2.2	9.74 ± 1.22	23.8 ± 3.4	10.6 ± 2.6
b. Stressed, .5 hr	8	1.67 ± .10	14.3 ± 3.2		23.7 ± 2.5	18.7 ± 6.2
c. " , 4 "	6	1.55 ± .08	14.5 ± 1.9		26.7 ± 5.1	37.0 ± 3.8
Lesioned: Habenula						
a. Non-stressed	7	—	16.5 ± 1.9	8.75 ± .72	25.2 ± 2.7	10.8 ± 3.3
b. Stressed, .5 hr	9	1.70 ± .07	15.6 ± 2.0		24.5 ± 1.2	27.0 ± 5.1
c. " , 4 "	7	1.52 ± .28	14.8 ± 2.0		25.2 ± 1.2	24.6 ± 3.6

All values represent mean ± stand. dev.
I^{131} uptakes were determined on all animals of each group 1 mo after lesioning.

group sacrificed 12-25 days after lesioning. No attempt was made using these preparations to define more closely the components of hippocampus or amygdala involved in the adrenocortical responses observed. Experiments are in progress using progressively smaller lesions in the amygdaloid nuclei to identify the region which exerts this modulating influence upon ACTH secretion.

Discussion. Although the role of afferent nervous impulses in ACTH secretion, including those from the limbic system, has been reviewed(24,2), the amount of experimental evidence is not great. Direct physiological and anatomical evidence for such a role(Mason, 14, 15; Nauta 25,26) is the observation that stimulation of the amygdaloid nuclei in the conscious, unrestrained monkey leads to elevated 17-hydroxycorticoid levels. Additional work by these investigators indicated that the adrenocortical response evoked by stimulation of amygdala or of hypothalamus may be inhibited by prior excitation of hippocampus. Porter(27) observed a similar inhibitory effect of hippocampal stimulation, using eosinophil response as an index of adrenocortical activation. In the dog and cat, Martin *et al.*(28) found elevated adrenocortical function after bilateral amygdalectomy; and Yamada and Greer(29) noted some increase in relative adrenal gland weights of rats 1-15 days after massive lesioning of the amygdaloid nuclei (but including hippocampus and lateral hypothalamus also), and suggested that this adrenal response was due either to non-specific stress or to destruction of a brain region which normally exerts some inhibitory influence upon ACTH secretion. In view of the short interval between lesioning and examination of the glands, the former possibility seems more likely to be correct.

Results of the present work add to the evidence that hippocampus and amygdala have a modulating influence upon the pituitary-adrenal axis, and suggest separate roles for these 2 components of the limbic system. The amygdaloid complex (or some portion thereof) is apparently required for acute, rapid activation of the hypothalamo-hypophyseal system; release of ACTH and adrenocortical response are not necessarily prevented, but are delayed following destruction of the amygdaloid nuclei. After 4 hrs of continuous immobilization, however, PFC levels were comparable to those attained by non-lesioned animals after 30 min or less of immobilization. On the other hand, chronically high resting PFC levels (without significant changes in adrenal weight) observed in rats with lesions of hippocampus indicate that this brain area normally contributes a suppressive influence to the milieu of afferent stimuli which reach the hypothalamus and maintain the basal or resting ACTH secretion.

Neural mechanisms which interplay upon the final source of ACTH-regulating neurohumor are both excitatory and inhibitory in nature; further experiments are necessary to determine the manner in which these neural influences are integrated under normal and stressful conditions into a resultant signal at the terminal cells responsible for the ACTH neurohumor.

Summary. Bilateral electrolytic lesions of hippocampus, amygdala or habenula of the rat did not produce notable changes in pituitary gonadotropic or thyrotropic activity as judged by testicular or thyroid weight and histology and I^{131} uptake. In response to immobolization stress, PFC levels of normal rats increased 200% after 30 min; after placement of lesions in amygdala, the response to stress was markedly delayed, but eventually reached a 200% increase after 4 hrs. Lesions of hippocampus did not alter the temporal pattern of response to stress; resting or basal PFC levels were, however, significantly higher than normal.

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Xanthine Oxidase Activity and Iron Storage in the Liver.*† (26833)

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It has been shown by others that xanthine oxidase participates in the release of iron from hepatic ferritin. The initial studies were carried out *in vitro* on rat liver slices subjected to lowered oxygen tension(1). With increasing activity of xanthine oxidase there was an increase in release of iron. Subsequent studies in intact animals supported this view(2). These authors suggested that ferritin iron was reduced to a more labile ferrous state making the iron available to iron binding agents such as transferrin. This then is reflected in the elevated plasma iron level.

If release of ferritin iron depends on xanthine oxidase activity it might be expected that decreasing the activity of this enzyme should result in accumulation of iron in the tissues. In the experiments to be described, liver xanthine oxidase activity was lowered by feeding sodium tungstate but there was no concomitant increase in liver iron stores.

Experimental procedure. Male albino rats of the Sprague Dawley strain, each weighing approximately 100 g, were fed a basic synthetic diet of the following composition: 65.7% glucose, 18% casein, 4% salt mixture(3), 11% corn oil, 0.3% choline chloride, and 1% iron citrate. To this diet were added crystalline vitamins in the following amounts per 100 g of diet: thiamine chloride 400 μg, pyridoxine hydrochloride 400 μg, riboflavin 800 μg, calcium pantothenate 1.5 mg, and nicotinic acid 2.5 mg. Each 100 g of diet was supplemented with approximately 60 U.S.P. units of Vit. A and 1 U.S.P. unit of Vit. D (Haliver oil, Parke-Davis). All animals were housed individually and given water *ad libitum*. The animals were divided into 2 groups of 16 each. In the experimental group the animals were fed the basic diet *ad libitum* supplemented with 16.84 mg of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) per 100 g of diet. The other group containing the pair fed control animals was fed the basal diet without sodium tungstate supplement. All rats were weighed twice a week. Hemoglobin and hematocrit determinations were performed at beginning and at termination of experiments. After 4 weeks the animals were sacrificed. The entire carcass was utilized for iron analysis in one half of each group. In these animals the intestines were removed, washed, and analyzed separately. The remainder of the animals in each group were autopsied and sections taken from liver, duodenum, pancreas, and pyloric portion of the stomach for histologic examination. Sections were stained with hematoxylin-eosin and for iron, using Perls' method. Livers were weighed and, after sections were taken for histologic examination, were analyzed for iron. Iron analyses were performed by the method of Kitzes, Elvehjem, and Schuette(4).

Liver xanthine oxidase was determined by the method of Remy, *et al.*(5) modified slightly by utilizing xanthine instead of hypoxanthine for the substrate. A 10% wt/vol suspension of each liver sample was prepared in 0.04 M phosphate buffer. For determination of enzyme activity 0.2 cc of the tissue extract was used. This represented 20 mg of the original liver sample. Enzyme activity was expressed as number of seconds required by the enzyme extracted from each 20 mg of liver to reduce the dye (Sodium 2,6-dichlorobenzenoneindo-3′-chlorophenol) from a colorimeter reading of 100 to one of 80. A Klett-Summerson colorimeter with a 660 mμ filter was used. In addition, a known amount of commercially available xanthine oxidase (Nutritional Biochemical Corp.) was tested by this method. Relationships between reduction time and enzyme concentration are shown in Fig. 1. When concentration of xan-

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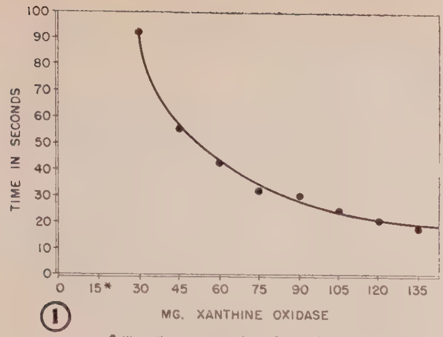
† Presented in part at 45th Annual Meeting of Am. Soc. Exp. Path., April, 1961.



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*When the concentration of xanthine oxidase was 15 mg. reduction time exceeded 300 seconds.

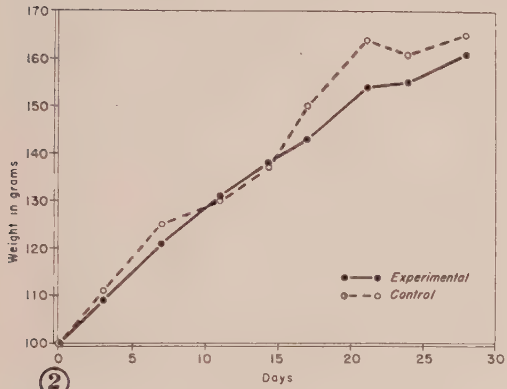

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FIG. 1. Relationships between reduction time and xanthine oxidase concentration.

FIG. 2. Avg weight changes in rats on a sodium tungstate supplement.

thine oxidase was 15 mg reduction time exceeded 300 seconds.

Results. The time required for dye reduction in the experimental group receiving the sodium tungstate was greater than 300 seconds per 20 mg liver (Table I). In comparison the time required for similar reduction in the pair fed control group ranged from 38 to 162 seconds with an average of 76 seconds. This indicated marked reduction in xanthine oxidase activity in the experimental group on the sodium tungstate supplement.

Liver iron values ranged from 0.98 to 1.83 mg per liver with an average of 1.25 mg in experimental animals and 0.85 to 1.49 mg with an average of 1.25 mg of iron per liver in pair fed control animals. These values were not statistically different when the 2 groups were compared ($p>0.1$). Further, when iron values were calculated as mg per 100 g of liver there was no significant variation. In addition, no differences were noted in amount and distribution of liver iron as determined histochemically. Occasionally small amounts of iron were seen in Kupffer cells and parenchymal cells in both experimental and control animals. When the entire carcasses were analyzed, experimental animals contained an average of 7.34 mg (6.40 to 8.52) and pair fed control animals contained an average of 7.65 (6.04 to 9.16) mg of iron per animal. As with the liver iron values, these values were not significantly different ($p>0.1$). There were no statistically significant differences in hemoglobin and hematocrit values when experimentals and controls were compared (Table I).

There was no significant difference in weight gain between experimental and the control animals (Fig. 2). Histologic changes were not seen in liver, pancreas, duodenum, or pyloric end of the stomach.

Discussion. It has been suggested that xanthine oxidase facilitates release of iron from liver *in vitro* by reducing the ferritin bound iron to the more readily dissociable ferrous states(1). If this enzyme is physiologically important in this role then the ability of the tissue to mobilize iron from its storage form would depend upon the level of activity of xanthine oxidase and a decrease of xanthine oxidase would be expected to give rise to an accumulation of iron in liver cells.

Our previous experiments showed that dietary ethionine decreased liver xanthine oxidase activity and increased iron deposition (6). Analysis of the covariates, total liver xanthine oxidase activity and total liver iron, showed a significant negative regression of liver iron values upon xanthine oxidase activity. This inverse relationship suggested

TABLE I. Effect of Tungstate Administration.

	Exp. group	Controls
Xanthine oxidase activity, sec /20 mg liver	>300	76
Total liver iron, mg	1.25	1.25
Liver iron, mg/100 g liver	21.9	22.4
Carcass iron, mg	7.34	7.65
Hemoglobin, g %	15.4	14.4
Hematocrit, %	51	47

that either the increase in liver iron values was caused by changes in xanthine oxidase or that both the changes in liver iron and xanthine oxidase activity were an expression of some other unidentified cause. In addition, statistical analysis indicated that the changes in xanthine oxidase could not have accounted for all the variation in liver iron values, and cast doubt on the hypothesis of a causal relationship between xanthine oxidase activity and iron deposition in liver *in vivo*.

To avoid the many effects of ethionine and still produce a lowering of xanthine oxidase activity, the present experiments were set up utilizing sodium tungstate(7) as a means of depressing xanthine oxidase activity. Under these circumstances there was a definite decrease in xanthine oxidase activity but no histologic changes in the animals. Neither was there any difference in weight gain. When total carcass iron values were compared, the animals with low liver xanthine oxidase activity had the same amount of iron in the body as the pair fed control animals without the lowered xanthine oxidase activity. This indicated that there was no difference in absorption of iron in both groups. Neither was there any alteration in storage of iron in the liver (Table I). In addition, there was no lowering of hemoglobin values in the experimental animals, indicating that they did not suffer from anemia as a result of possible decrease in release of body storage iron. It was concluded from these studies that the lowering of liver xanthine oxidase activity did not affect iron deposition in the liver or iron absorption. Recently Strohmeyer, *et al.*(8) utilizing a different approach failed to demonstrate a relationship between liver xanthine oxidase activity and iron metabolism. It would seem from our study that whatever role the liver xanthine oxidase may play in iron metabolism it has no direct effect on liver iron storage or on iron absorption.

Summary. Sodium tungstate was added to the diet to decrease liver xanthine oxidase activity. There were no concomitant changes in liver iron or iron absorption associated with the decrease in xanthine oxidase activity.

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Changes in Total Peripheral Resistance in Endotoxin Shock.* (26834)

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Previous reports have described changes in total peripheral resistance during irreversible hypotension following injection of endotoxin(1-5). Results, however, have differed and the question of resistance changes in endotoxin shock is not settled. Two general procedures have been previously employed for calculation of vascular resistance: (a) measurements of arterial pressures during constant cardiac inflow in perfused animals (3,4), and (b) measurements of arterial pres-

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sure and cardiac output in intact animals administered endotoxin(1-3). These procedures are considered inadequate for several reasons: in the first instance, maintenance of a constant cardiac inflow during the post-endotoxin period may prevent significant alterations in resistance associated with neurohumoral or physical changes precipitated by severe hypotension(6-10). In the second instance a major difficulty arises in interpretation of resistance changes because of the tendency for resistance to increase as flow and distending pressure decrease(6,7). Since calculated total systemic resistance has been found to be more uniformly related to flow than to pressure(7), the present study was devised to control the parameter of flow (cardiac output). This study is considered important insofar as it provides a possible explanation for the development of irreversible shock.

Materials and methods. Studies were performed on 16 adult dogs weighing between 7.0 and 12.3 kg, anesthetized with sodium pentobarbital, 30 mg/kg. Animals were respired with room air provided by a constant pressure respirator. Aortic pressure was measured through a catheter inserted into the femoral artery connected to a Statham strain gauge and registered on a Sanborn direct writing recorder. A reservoir system was interposed between the central ends of the great veins (azygous vein ligated) and the right atrium, thus allowing direct measurement of the venous return. The system was primed with homologous heparinized blood. A sigmamotor pump returned the blood from the external reservoir to the right atrium. This procedure has been previously reported(3,4). Following cannulation of the right atrium and great veins the atrial inflow rate was repeatedly adjusted by altering the sigmamotor pump speed to maintain the venous reservoir at a constant volume. Within 30 minutes equilibrium was established and a 20 minute control period was begun. Flows during this period averaged 77 cc/min/kg body weight (range, 66-88). Cardiac inflow is assumed to equal cardiac output, under the conditions of these experiments(3,4). Venous return was periodically measured with cylinder and stop watch and a control period of constant flow was established for each preparation. Animals were divided into 3 groups: the first group consisted of 5 control animals in which the right atrial inflow exactly matched the venous return. The second group was composed of 6 dogs given a lethal dose of *E. coli* endotoxin‡ (0.55 mg/kg). In these dogs, as in the first group, the sigmamotor pump speed was varied so that venous return and cardiac inflow were equal. In a third series of 5 dogs endotoxin was not administered but the right atrial inflows were adjusted to approximate the average flows of the experimental group administered endotoxin. All experiments were terminated at 75 minutes beyond the control period due to development of excessive bleeding from cut surfaces, which interfered with the accuracy of the measurements.

Results. Fig. 1 illustrates the results from 5 dogs serving as controls and not administered endotoxin. There was no change in total peripheral resistance during the perfusion period although there was a gradual fall in both cardiac inflow and mean systemic arterial pressure. The results from this series served to evaluate the experimental procedure.

Fig. 2 provides mean values for 6 animals given endotoxin. Resistance remained fairly constant during the 35 minute period after endotoxin. Beyond this time there was a steady fall in resistance. Pressure and flow fell steadily until about 10 minutes after endotoxin, then rose to maximal values at about 25 minutes. Following this there was a steady drop in pressure with the cardiac inflow remaining relatively constant.

Fig. 3 gives results from 5 dogs not administered endotoxin, but having cardiac inflows matched with the mean flows of animals receiving endotoxin. Resistance rose following the initial reduction in flow and remained elevated until termination of the experiments. The drop in pressure after 25 minutes was more gradual than that shown in Fig. 2 which accounts for the differences in resistance between the latter 2 groups.

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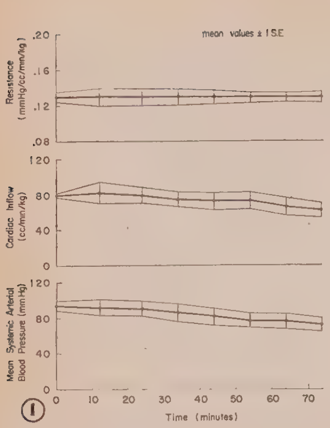
‡ Difco endotoxin.



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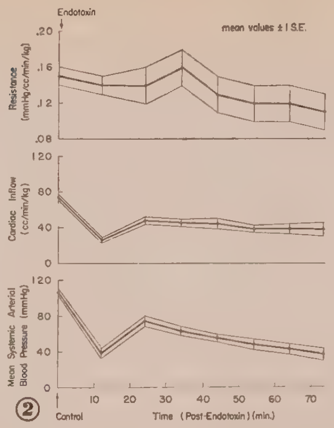

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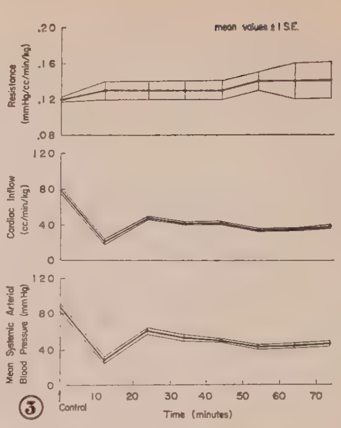

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FIG. 1. Control experiments, showing changes in total peripheral resistance, cardiac inflow and mean systemic arterial blood pressure in 5 perfused dogs.

FIG. 2. Changes in total peripheral resistance, cardiac inflow and mean systemic arterial blood pressure in 6 perfused dogs administered lethal doses of endotoxin.

FIG. 3. Changes in total peripheral resistance, cardiac inflow and mean systemic arterial blood pressure in perfused dogs with artificially reduced cardiac inflows but not receiving endotoxin (see text).

Discussion. Evaluation of experimental technic. The procedure described appears to be useful in application to the general problem of total peripheral resistance changes in shock. The attempt was made (Fig. 3) to control the flow component of resistance(6,7) in order better to interpret the resistance changes at low cardiac inflows in animals administered endotoxin. Advantages of the procedure are the relative simplicity of the operative and perfusion technics and the convenient determinations of both flow and resistance. *Evaluation of results.* Findings show that the initial fall in blood pressure in dogs administered endotoxin is primarily due to a decrease in venous return. This is consistent with data obtained from constant cardiac inflow experiments(3). Following this period arterial pressure rises to a maximum at about 25 minutes, which is assumed to represent the period of "compensation", and appears to be brought about by an increase in venous return (Fig. 2). The data suggest that this maximal increase in mean systemic arterial pressure marks the onset of the irreversible phase of endotoxin shock, characterized by a progressive fall in total peripheral resistance and a steady decline in arterial pressure (Fig. 2). It has been reported(6,7) that resistance tends to increase as flow and distending pressure decrease, and on this basis one would expect that the resistance would rise in the later phase of endotoxin shock. That this did occur in animals not given endotoxin but whose cardiac inflows were artificially diminished (Fig. 3) indicates that the decline in peripheral resistance in animals in endotoxin shock assumes a more prominent role. It is apparent from this study that the vascular response of the endotoxin animals is inadequate to support the systemic arterial pressure, and that a decrease in vessel tone has occurred. Recent findings in this laboratory(8-10) suggest that both neural and humoral factors may be responsible for the fall in systemic resistance.

Summary. A series of experiments has been carried out on dogs to determine total peripheral resistance changes in endotoxin shock. Animals were perfused with homologous heparinized blood at controlled and measured cardiac inflows. Results suggest that the onset and course of irreversible shock are characterized by a progressive fall in total peripheral resistance. The mechanism of the decline in resistance has been suggested by earlier studies from this laboratory.

Appreciation is expressed to Margaret M. Jordan for technical assistance.

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Effects of Pretreatment with Antibiotics on Survival Following Mesenteric Arterial Occlusion in Rats. (26835)

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Recent studies have shown that changes in the intestinal tract are largely responsible for the irreversible course of the syndrome initiated by hemorrhagic, traumatic, and septic shock. Fine *et al.*(1,2) and Lillehei(3) have provided evidence indicating that the primary factor in this regard in the rabbit and dog is the systemic dissemination of bacterial products derived from the intestinal flora.

It appeared to us that the shock induced by temporary obstruction of the blood flow to the intestinal tract(3) would serve as an excellent test for this thesis. In particular, the reported ability of antibiotics(1,4) to afford protection by circumventing the usual intestinal bacteremia, required investigation in this type of shock.

Experiments in the rat reported herein do not support the concept of a systemic bacterial endotoxemia in bowel occlusion shock, but point rather to local factors within the intestine proper as the decisive elements in the fatal course of the syndrome.

Materials and methods. Two strains of rats, totaling 119 animals of both sexes, were used. These were German albino rats weighing 105 to 205 g and NAMRU stock rats weighing 110 to 300 g. In addition, 30 rats, which were excluded from the survival data, were used for observation of omental circulation, for measurements of mean arterial blood pressure, and for bacterial cultures. All rats were anesthetized with sodium pentobarbital (3 mg/100 g body weight, i.m.).

Shock was induced by temporary occlusion of the circulation to the intestinal tract. A midabdominal incision was made through the *linea alba* to minimize bleeding and the superior mesenteric artery was isolated. The artery was occluded with a wire serrafine clamp for 2 hours, and then released. The wound was closed and the rat returned to its cage. Aseptic precautions were taken throughout. Only those animals which survived the procedure are included in the data. Survival statistics are based on a 24-hour period of observation. Autopsies were performed routinely.

In representative members of each group of experimental animals, the blood was heparinized and mean arterial blood pressure was measured in the left carotid artery by a cannula leading to a conventional mercury manometer. Circulation of the omentum was also observed in these animals using Zweifach's visualization technic(5). Bacterial cultures (aerobic and anaerobic) were made on liver, spleen, intestine, and cardiac blood on selected animals (Table II).

Three series of experiments were conducted. In the first series, 6 groups of rats (77 animals) were given antibiotics. Five different antibiotics were employed, to pro-

TABLE I. Effect of Pretreatment with Antibiotics on Shock Following Occlusion of Superior Mesenteric Artery.

Agent	Amount*	Duration	Survival†		Strain
				(%)	
Controls—Water	.8 ml	6 days	4/18	22	German
"	.8 "	6 "	8/24	33	NAMRU
Terramycin	100 mg	6 "	8/11	73	German
"	100 "	Single dose	1/6	17	"
"	+ 2 " ‡	" "			
Gantrisin	.25 g	6 days	8/10	80	"
Aureomycin	100 mg	6 "	4/16	25	NAMRU
Neomycin	100 "	6 "	11/14	79	"
Chloromycetin	100 "	6 "	10/20	50	"

* Both water and antibiotics were given orally, in a volume of 0.8 ml.

† The significance of the survival data following antibiotic treatment was tested by the chi square method using Yates' correction factor. Only Terramycin, Gantrisin, and Neomycin values are statistically significant at 5% level ($\chi^2 > 3.841$).

‡ In 0.2 ml saline, inj. into portal vein following occlusion.

vide as wide an anti-bacterial spectrum as possible and to avoid the difficulty of possible pharmacological effects of particular substances over and above their antibiotic potentialities. Two of the materials (Aureomycin and Neomycin) have been previously found to protect dogs against a normally lethal episode of hemorrhagic shock(1). Terramycin and Gantrisin in general have a range of anti-bacterial effectiveness similar to the above 2 agents. Chloromycetin was included because it is particularly effective against gram-negative bacteria.

The animals were force fed by stomach tube 100 mg (in 0.8 ml water) of the following antibiotics for 6 days: Terramycin, Aureomycin, Neomycin, and Chloromycetin. Gantrisin (3,4-dimethyl-5-sulfamido-isoxazole) was given in 0.25 g doses. The animals were subjected to shock on the sixth day. Two control groups (42 animals) received only water. The 6-day period of treatment is similar to that employed by others(1,4) in the dog.

One group of animals received 100 mg Terramycin *per os* several hours before and 2.0 mg of antibiotic into the portal vein just after the superior mesenteric artery had been released (Table I).

The second and third series of experiments dealt with 2 forms of tolerance. In the second series, rats (20 animals) were made resistant to drum trauma by exposing them to progressively increasing numbers of rotations in the Noble-Collip drum(6). Resistance was induced by subjecting the rats on the first day to 100 rotations, on the third day to 200, on the fifth day to 400, on the seventh day to 500, and on the ninth day to 900 rotations. The rats were then subjected to bowel occlusion shock on the following day.

In the third series, 19 rats were made tolerant to endotoxin by serial intraperitoneal administration of *E. coli* endotoxin (Difco) beginning with 10 μg on day 1 and then at successive daily intrevals 20, 40, 80, 150, and 250 μg in 0.5 ml saline. The rats were subjected to the same occlusion shock 24 hours later. A group of 20 control rats received only saline (0.5 ml, i.p.) for a similar period.

Experimental findings. 1. *Survival.* The data (Table 1) show that rats pretreated with Terramycin, Neomycin, or Gantrisin had the highest survival rates, 73%, 79%, and 80%, respectively, in contrast to the considerably lower survival rate of the controls (22% and 33%). It should be noted that Neomycin, one of the 3 effective antibiotics, is poorly absorbed, supporting the contention(1) that the protective effect of such pretreatment is due in the main to a local antibacterial action within the bowel proper. Unlike the reported efficacy of Aureomycin in hemorrhagic shock(1), no beneficial effect was observed against bowel occlusion shock. The Chloromycetin data were not consistent—in one run 3/10 survived, in another 7/10. The rats which received Terra-

TABLE II. Bacteriologic Findings.

Group	Rat No.		Liver	Spleen	Blood	Intestine
	43	No occlusion	—	—	—	E, Sa, Sta, Str, Sub, Y
	44	*Idem*	—	—	Sub	A, B, E, Sa, Sta
Control	40	Immed. after release	B, Sta, Sub, E	E, Sta	B, Sta	E, Sa, Sta, Pr
	41	*Idem*	B, Sta	—	Sta	B, E, Pr, Ps, Sta
	42	”	—	E	—	E, Pr, Ps, Str
	36	”	—	—	—	E, Pr, Ps, Str
Terramycin	37	”	Sta	—	B, Sta	E, Pr
	47	24 hr after	E, G, Sub	E	B, N, Sub	E, G, Pr, Ps, Sta
	38	Immed. after release	—	—	—	E, Sta
Gantrisin	39	*Idem*	—	—	—	B, E, Pr, Sta, Sub
	45	24 hr after	E, Ps, Sub	E, Pr, Sub	N, Pr, Sub	E, Pr, Ps, Sta
	46	*Idem*	E, Pr, Ps	B, E	B, E	B, E, Sta, Pr

Stool of #43: E, Pr, Sh, Sa.

Key: A = *Aerobacter aerogenes,* B = Bacteroides, E = *Escherichia coli,* G = *Gaffkya tetragena,* N = Neisseria, Pr = Proteus, Ps = *Pseudomonas aeruginosa,* Sa = Salmonella, Sh = *Shigella dysenteriae,* Sta = Staphylococcus, Str = *Streptococcus* sp., Sub = *Bacillus subtilis,* Y = Yeast.

mycin on the day of experiment survived less well than the controls (17%).

The 2 types of induced tolerance studied were found to have no significant protective action (40-42% survival). Here again, this finding is not in agreement with previous reports(1) on hemorrhagic and drum shock in rats where endotoxin and drum adaptation made the animals resistant to these forms of stress.

2. *Blood pressure.* The mean arterial blood pressure in fatal cases fell to shock levels (below 40 mm Hg) 2 hours after the clamp was released. The blood pressure rose slightly at time of occlusion to 150 mm Hg and remained at this level throughout the period of occlusion. Immediately upon releasing the clamp, the blood pressure dropped only slightly, about 10-25 mm Hg. In the antibiotic protected rats, the pressure rarely fell during the next 1-2 hours to below 100 mm Hg, whereas in the control group the pressure continued to decline during the initial 60-90 minutes and remained below 70 mm Hg. The rapid drop in pressure to 30 mm Hg occurred during the final 30 minutes of life.

3. *Bacteriology.* The results of bacterial cultures are summarized in Table II. Tissue and blood specimens from both control rats and those pretreated with antibiotics showed no growth (under aerobic and anaerobic incubation) when taken immediately after release of the clamp, except for samples from the intestines and stool. In contrast, all specimens taken at 24 hours after arterial occlusion were uniformly positive. Rats which survived were quite active and alert despite the positive bacteremia. There were no essential differences in the bacterial populations of unprotected controls which died and antibiotic protected rats which survived. Exact quantification was not attempted, since it was felt that by demonstrating the presence of gram-negative organisms, the point at issue was proved. It should be noted that bacterial species such as *Proteus, Bacteroides,* and *Pseudomonas* were uniformly present after shock, whereas they were either not encountered or present only in minor numbers in controls. The antibiotic regimen, although adequate for protection against lethal shock, did not significantly suppress the bacterial population in the intestinal lumen; nor was the incidence of bacteremia in blood, liver, and spleen after shock different in controls than in antibiotic treated rats.

4. *Pathological changes.* During the period of occlusion, the small intestines assumed a somewhat grayish or bluish appearance in all of the groups. Some of the antibiotic treated animals (especially those given Neomycin), showed a tendency toward intestinal edema. Upon release of the arterial

clamp, the intestine of the controls assumed a reddish, patchy appearance, whereas in the groups protected by antibiotics (Terramycin and Gantrisin), as well as after Aureomycin, the gut became hyperemic and showed no evidence of intraluminal hemorrhage.

In animals which succumbed, whether they had been treated or not, lungs were congested, adrenals enlarged, liver swollen and dark, and the spleen dark. Blood was usually found in the intestinal lumen. It was of interest that the endotoxin tolerant animals following bowel occlusion shock showed to a much lesser degree any of the above pathological changes. In particular, the intestinal tract had a more normal appearance. Drum tolerant rats showed an especially severe intestinal necrosis.

Upon sacrifice, animals which had survived, because of pretreatment with antibiotics, showed few or no abnormal findings, except for minor congestion of the lungs (probably the result of anesthesia).

5. *Omental observations.* Few changes either in distribution of blood, velocity of flow, or abnormal vasomotor behavior were seen during the period of arterial occlusion. Blood flow in the omentum was apparently maintained by the gastric branches of the celiac artery. After release of the arterial clamp, overall blood flow gradually diminished, the velocity of flow in the collecting venules being especially slowed within 45-60 minutes. Two hours after release of the clamp, there was no active circulation in most of the capillaries. The venules developed perivascular collars of hemorrhage and extensive stasis was present in the effluent capillaries.

In the Terramycin group, blood flow remained active throughout the 3-hour period of observation after clamp release. There was much less evidence of the viscous type of flow seen in fatal cases. Perivascular hemorrhage did not develop to any appreciable degree and capillary stasis was absent. Of the 4 animals observed in the antibiotic treated group, only one showed evidence of capillary stasis after 3 hours.

Discussion. There is good evidence that factors originating in the small intestines contribute to the circulatory derangement characteristic of various types of shock, including strangulation of the bowel(7-10). It has been suggested(11) that the bacterial flora in the intestines give rise to toxic products which, in the face of an impaired defense mechanism during protracted shock, are disseminated systemically and interfer with peripheral circulatory homeostasis. Apparently, in cases of intestinal strangulation, a lethal fluid is exuded(12) which is heavily infected with many of the bacterial flora present in the bowel.

The bacterial cultures in the present studies of bowel occlusion shock indicate that bacteremia *per se* may not be directly concerned with the lethality of the syndrome since even those rats which survived showed positive blood and tissue cultures 24 hours later.

Terramycin and Gantrisin (2 absorbable agents) and Neomycin (a non-absorbable agent) clearly showed a protective action. On the other hand, Aureomycin and Chloromycetin were not as effective. The basis for this distinction is not clear. Rats which had been pretreated with the various antibiotics uniformly showed less severe pathological changes in the intestines, despite the fact that 2 of the groups (Aureomycin and Chloromycetin) were not protected.

Although drum resistant rats have been found more tolerant of acute hemorrhagic and traumatic shock(13), they were not more resistant to bowel occlusion shock. Evidently, different mechanisms are involved in these several forms of shock. Rats made tolerant to endotoxin have been shown to withstand normally lethal episodes of hemorhagic and traumatic shock(14). They were not, however, able to tolerate the bowel ischemia following occlusion of the superior mesenteric artery. This circumstance by itself argues against endotoxemia as the major factor in the collapse of the circulation following bowel occlusion.

Passive transfer experiments(15) indicate that the blood of rabbits contains a lethal factor during shock after occlusion and release of the superior mesentric artery. However, as in the current study, this earlier report was unable to substantiate the presence of endotoxic material in the blood stream during this type of shock.

Blood pressure masuremnts were not especially informative. Blood pressure dropped more abruptly in controls following removal of the arterial occlusion than it did in antibiotic protected animals. Apparently, the bowel was better able to withstand circulatory arrest when bacterial flora was suppressed by antibiotics. Subsequently, blood pressure fell progressively in those rats which ultimately died, but remained at near normal levels in survivors. These differences may be due to absence of swelling and plasma loss in the intestinal tract of antibiotic treated rats. Direct visualization of the omental circulation supports this possibility since the blood flow in protected animals did not show the hemoconcentration exhibited by the controls. Venular stagnation and precapillary hemorrhage, which were prominent features of the mesenteric circulation in fatal shock, did not develop to any significant extent in antibiotic protected animals.

In general, the protection afforded by orally administered antibiotics would appear to be a local circumstance which minimizes the pathological changes in the intestinal wall during shock and thereby avoids the attendant plasma loss and frank hemorrhage which are a prominent feature of bowel ischemia shock.

Summary and conclusions. Shock induced in rats by temporary occlusion of the superior mesenteric artery after pretreatment with various antibiotics and induction of resistance by drum training and by repeated administration of bacterial endotoxin was studied. Terramycin, Neomycin, or Gantrisin given orally for 6 days clearly protected rats, whereas those treated with Aureomycin or Chloromycetin were not protected. Adaptation to drum trauma did not circumvent the lethal effects of bowel occlusion. Tolerance to bacterial endotoxins was only moderately effective against occlusion shock.

It was concluded that the protective action afforded by certain antibiotics was probably due to their local action in preventing pathological changes in the intestinal tract and not to prevention of a systemic bacteremia.

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Effect of Metal Ions on Brain Peptidase Activity.[*] (26836)

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The substrate specificity of mouse brain peptidase activity toward a wide variety of peptides representing different structural arrangements of constituent amino acids has been demonstrated(1,2). This specificity was present without addition of any co-factors or activators. The present study concerns itself with the effect of some biologically significant metal ions on cerebral peptidase activity towards 2 representative peptides. The first of these, glycyl-L-leucine, represents the prototype of N-glycyl dipeptides with an aliphatic (or aromatic) lipophilic side-chain adjacent to the terminal carboxyl group which is maximally split by the cerebral peptidase system(2). The second, triglycine, represents a class of N-glycyl tripeptides in which the N-glycyl residue is split independently of the other residues(2).

Materials and methods. The mice used were Swiss albino mice from the colony maintained at the Harvard Medical School. Extensive chemical and histological data on the brain of this strain were already available (3). Fresh peptidase preparations in 30% glycerol in 4% phosphate buffer (0.067 M) at pH 7.0 were prepared for each experiment in the manner described in detail earlier(2), to yield 1.0 mg of dry tissue equivalent enzyme per 50 μl of extract. The metal ions Cu^{++}, Zn^{++}, Cd^{++}, Mg^{++}, Ca^{++}, Mn^{++}, $MoO_4^{=}$, Fe^{+++}, Co^{++}, Al^{+++}, Ni^{++} and Hg^{++} were added to the enzyme extract in the form of $CuSO_4$, $ZnSO_4$, $CdCl_2$, $MgSO_4$, $CaCl_2$, $MnCl_2$, Na_2MoO_4, $FeCl_3$, $CoCl_2$, $AlCl_3$, $NiCl_2$, and mercuric acetate respectively. All metal salts were analytical reagent grade. Concentration of metal ion was varied (1-5 μg metal/mg dry tissue equivalent enzyme), while the volume added was kept constant at 4 μl. Thorough mixing of the metal ions with the peptidase preparation was achieved by briefly "buzzing" the microtubes on the shaft of a micromotor. The peptide substrates (5 μM peptide in 50 μl volume) were then added. Incubation and formol titration were performed as described in detail previously (2). The purity of the peptides used was rigidly controlled chromatographically(2), and as an added precaution the triglycine was recrystallized twice from boiling water and ascertained to be chromatographically pure in 2 different solvent systems. Every experiment had parallel duplicate blanks for enzyme alone, enzyme plus metal, and metal plus peptide which were titrated in the same experiment. Blank values were subtracted from the test values. In no case did the presence of the metal ion, within the concentration range used, interfere with titration of liberated carboxyl groups.

Results. The effect of the different metal ions on hydrolysis of glycyl-L-leucine by cerebral peptidases is presented in Fig. 1. The results are expressed as percent of the hydrolysis of peptide without added metal ions, this value being 100%. These values are plotted against the different amounts of metal ions present (in moles). It is immediately apparent that the inhibitory effect of metal ions on the enzymatic hydrolysis of glycyl-L-leucine by brain is different for most divalent metals tested. Mercury, cadmium and copper ions, in that order, showed highest inhibition of glycyl-L-leucinase activity. The effect of Zn^{++} was intermediate between this group and Mn^{++} and Co^{++} whose inhibition never was more than 40%. Fe^{+++}, Al^{+++}, Ca^{++}, Mg^{++}, Ni^{++} and $MoO_4^{=}$ were without effect on this system except at very high concentrations (10-20 $\mu M \times 10^{-2}$ Metal/mg dry tissue equivalent enzyme) in which range they produced non-specific precipitation of protein in the incubation mixtures. The effect of metal ions on the hydrolysis of triglycine (Fig. 2) was more complex in view of the fact that the hydrolysis of 2 peptide

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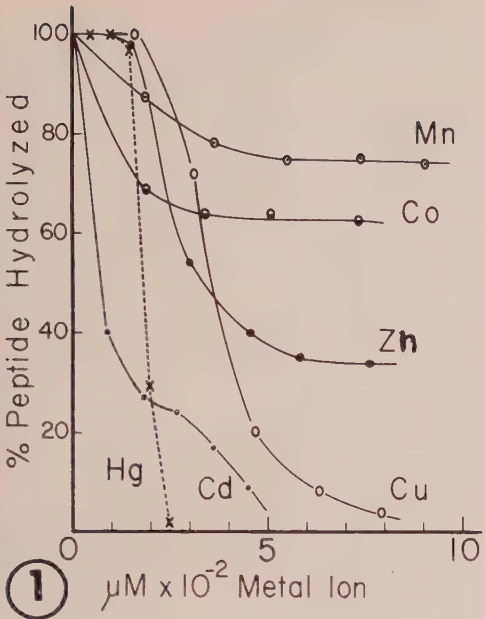
[*] Supported by Research Grant from U.S.P.H.S.



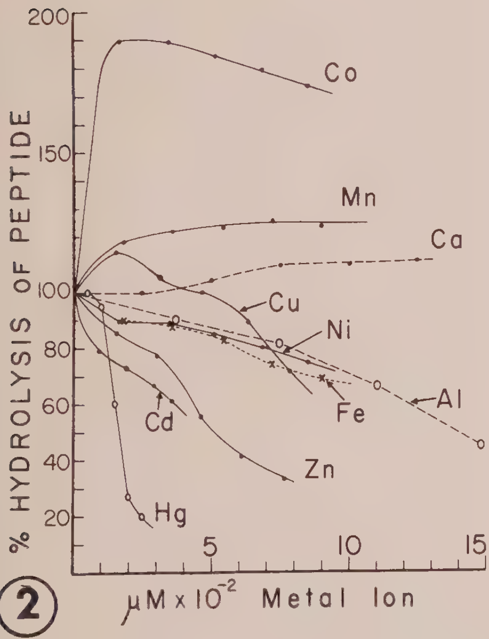


FIG. 1. Inhibitory effect of Hg^{++}, Cd^{++}, Cu^{++}, Zn^{++}, Co^{++} and Mn^{++} on hydrolysis of glycyl-L-leucine by cerebral peptidases. Ni^{++}, MoO$_4^=$, Fe^{+++}, Al^{+++}, Ca^{++} and Mg^{++} were without effect.

FIG. 2. Effect of metal ions on the enzymatic hydrolysis of glycyl-diglycine. Co^{++} ions activate hydrolysis of the diglycine residue.

bonds was involved. The hydrolysis of the N-terminal glycine residue which is hydrolyzed without metal ions(2), was inhibited maximally by mercury, cadmium and zinc ions, in that order. Copper, nickel, trivalent iron, and aluminum ions exhibited less pronounced inhibition (less than 50% at 10 µM $\times$ 10^{-2} Metal ion/mg dry tissue equivalent). Molybdate and magnesium ions were without any effect. At low concentrations of copper (0.002 µM/mg dry tissue equivalent) there was minimal splitting of the diglycine residue. Manganese ions produced a similar effect in all concentrations tested. The effect of Co^{++} was most impressive in that they produced a splitting of both peptide bonds leading to almost complete hydrolysis of triglycine to 3 moles of glycine. This was confirmed by paper chromatography of the end-products of enzymatic hydrolysis of triglycine in the presence of cobalt ions. The hydrolysis of diglycine alone, which is minimal in the absence of added activator, was shown to proceed to 90% completion on addition of extremely small amounts of cobalt (Fig. 3).

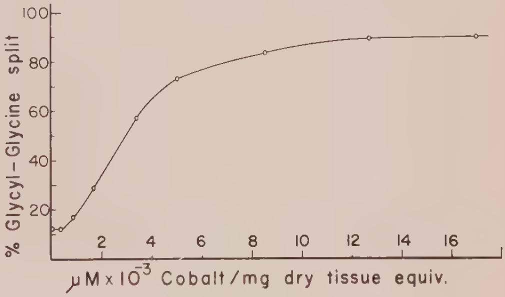


FIG. 3. Activation of cerebral diglycinase by cobalt ions.

In view of the fact that diglycine represents the first in the series of N-glycyl dipeptides with no aromatic or aliphatic non-polar side chain adjacent to the free carboxyl group, and because this group of peptides had been shown not to be split by cerebral peptidases (2), the effect of cobalt ions on the hydrolysis of other members of this group was tested. When glycyl-γ-aminobutyric acid, glycyl-L-aspartic acid, and glycyl-L-glutamic acid were incubated with enzyme, with and without addition of different concentrations of Co ions, no increase of hydrolysis could be demonstrated by titration or chromatography. It therefore appears that the effect of cobalt is specific for hydrolysis of the dipeptide glycyl-glycine.

Discussion. Any attempt at quantitating the inhibition of a metal ion on an enzymatic system in which the bulk of the protein is probably not the enzyme, as in our case, has to make allowance for the fact that a certain proportion of the metal added will be non-specifically bound to inert protein, and only a portion will be free to react with the enzyme or the substrate. This non-specific binding leads to greatest errors in low metal concentration and is of less importance at higher concentration. The curves depicting the inhibitory effect of metals on enzymatic hydrolysis of glycyl-L-leucine and triglycine (Figs. 1 and 2) are therefore open to question as to whether inhibition actually does occur with much lower concentrations of each of the metal ions studied. This cannot be answered until each of the cerebral peptidases can be tested in pure form. The extremely low concentrations of cobalt ions needed to activate diglycinase activity would tend to argue against any significant portion of this metal being non-specifically bound by inert proteins. The data do show that there is a relative difference in the inhibitory effects of the different metals, some like Hg^{++} and Cd^{++} being more potent inhibitors by several orders of magnitude. The effect of cobalt ions on the hydrolysis of the diglycine residue of triglycine after the N-terminal glycine is removed by a separate enzymatic system strongly suggests that the cerebral enzyme responsible for diglycinase action is very similar to that described by Smith(4) in other tissues, and as such represents a distinct and separate enzyme in the mixture of enzymes operating in our cerebral peptidase system. The very low but measurable diglycinase activity without addition of cobalt observed here (Fig. 3) and in earlier studies (2) probably reflects the activity resulting from the pre-existing tissue cobalt. It is anticipated that extension of these studies to other peptidase systems may provide clues to the nature of the "toxic" effects of some of the metal ions studied here in disase processes like Wilson's disease, lead and mrcury poisoning(5,6).

Summary. The effect of Ca^{++}, Mg^{++}, Cu^{++}, Zn^{++}, Cd^{++}, Co^{++}, Mn^{++}, MoO$_4^{=}$, Ni^{++}, Fe^{+++} and Al^{+++} was studied on the enzymatic hydrolysis, by cerebral peptidases, of glycyl-L-leucine and glycyl-diglycine. The inhibition was shown to be of different orders of magnitude for different metals. Only cobalt ions were shown to enhance the hydrolysis of glycyl-diglycine by promoting the hydrolysis of the diglycine residue in the tripeptide which normally resists hydrolysis. This effect was specific for diglycine and not applicable to the other N-glycyl dipeptides which are normally not hydrolyzed by brain.

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Lack of Relationship Between Sialic Acid Content, Toxicity, and Lethality of *Escherichia coli.* (26837)

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Some strains of *E. coli* are more lethal than others for mice. The reason for this difference is not known. The possibility that sialic acid which occurs in cells of some strains may be involved in their lethality was suggested by the following considerations:

Kauffmann(1) found that the presence of certain L antigens, which occur as capsules or envelopes, was associated with increased virulence. Recently, Barry(2), in a survey of various strains of antigenically different *E. coli,* found sialic acid in certain strains with L type antigens, and not in strains with A or B type antigens. The question thus was raised: could sialic acid be related to those L type antigens whose presence had been associated with increased virulence?

We have tested the hypothesis, mentioned previously by others(3), that sialic acid in *E. coli* may play a role in enhancing their virulence or toxicity. Virulence was measured as lethality of viable cells for mice and toxicity was measured by the lethal effects of killed cells. We have further determined sialic acid content and virulence of young and older cells of the same strain and compared the toxicity of extracts from such cells.

Method. The study was carried out with 13 strains, each of a different serological type.* The cultures were maintained on nutrient agar slants and stored at 4°C.

The general procedure was as follows: 200 ml batches of trypticase soy broth (Baltimore Biological Labs.), inoculated with 2 ml of an overnight broth culture, were incubated for 5 hours at 37°C at which time the culture was in the upper half of its logarithmic phase. In some cases, cultures were incubated for 24 hours. An aliquot was removed and used for lethality titrations, and the remainder was centrifuged. The cells were washed once with distilled water, suspended in water and lyophilized.

For lethality titrations one-half ml portions of serial dilutions in broth of the cultures grown for 5 or 24 hour periods were injected intraperitoneally in male CF1 (Carworth Farms) mice weighing 20 ± 2 g each. The number of organisms injected was determined by plate count. Ten mice were used per infecting dose and mortality noted over a period of 7 days. Titrations were performed 3 or 4 times with each strain. LD_{50} values of the individual tests, expressed as number of viable cells, were determined using an electronic computer programmed following procedures given by Finney(4). The computed values were combined to give a pooled LD_{50} for each strain.†

For toxicity titrations a weighed amount of lyophilized cells was suspended in saline and killed by exposure to 100°C for 10 minutes. One-half ml of dilutions of these suspensions was injected intraperitoneally in mice. Ten mice were used per dose and mortality was observed over a period of 7 days. Toxicity titrations were performed 2 or 3 times for each strain. LD_{50} values of the individual tests, expressed as mg of heat killed cells, were determined and combined to give a pooled LD_{50} for each strain.

Due to interference by chromogenic materials, it was not possible to apply colorimetric tests for sialic acid directly on the lyophilized cells. Sialic acid determinations were, therefore, carried out on hydrolysates of lyophilized cells according to the procedure of Svennerholm(5). Preliminary tests showed that no loss of sialic acid occurred in washing and lyophilizing of cells. Hydrolysates were prepared by suspending lyophilized cells (3 to 15 mg/ml) in 0.1 N H_2SO_4 and maintaining the suspensions at 80°C for one hour after which cellular material was removed by centrifugation. Preliminary tests had shown, in agreement with the findings of DeWitt and Rowe(3), that this hydrolysis procedure was optimal. Hydrolysates of 10 to 100 mg of cellular material were passed over a Dowex 2-X-8 resin (acetate form) column. After washing the column with water the adsorbed sialic acid was eluted with 8 ml of 1 M sodium acetate-acetic acid buffer (pH 4.6), followed by a 6 ml aliquot of buffer. The column eluates were tested for sialic acid by the resorcinol method(5) using N-acetylneuraminic acid as standard, and results were expressed in terms of this compound. Deter-

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* Strains of *E. coli* were kindly provided by Dr. W. H. Ewing (Communicable Disease Center, Chamblee, Ga.), Dr. C. W. DeWitt (Upjohn Co.,), Dr. G. T. Barry (Univ. of Tennessee), and Dr. M. K. Nadel (Lederle Laboratories). We wish to thank Dr. W. H. Ewing, for verifying the serological type of several strains.

† Statistical computations were carried out by Dr. W. Kwolek (Statistical Dept., Lederle Laboratories).

TABLE I. Lethality, Toxicity and Sialic Acid Content of *E. coli* of Various Serotypes.

Strain and origin	LD_{50} for mice, No. viable organisms/mouse × 10^5	Relative virulence*	LD_{50} of heat killed cells, mg/mouse	Sialic acid, μg/mg cells, avg and range†
$O_{16}K$- (Ewing)	1100 (840-1400)‡	1	2.7 (2.2-3.3)‡	<.3
$O_{111}B_4$ (Nadel)	800 (220-1600)	1	2.4 (1.9-2.9)	<.3
$O_{127}B_8$ (")	790 (620-1000)	1	1.5 (1.0-2.6)	<.3
$O_{55}B_5$ (")	370 (260- 530)	3	1.6 (1.0-2.7)	<.3
$O_{16}K_1$ (Ewing)	360 (130-1000)	3	3.9 (3.4-4.5)	11 (8-17)
O_9K_{29} (")	330 (150- 730)	3	2.1 (1.0-2.5)	<.3
O_9K_{35} (")	180 (140- 240)	6	1.2 (.8-1.5)	<.3
$O_{26}B_6$ (Nadel)	92 (60- 140)	12	1.5 (1.2-1.8)	<.3
$O_{10}K_5$ (Barry)	15 (12- 20)	70	1.5 (1.2-1.9)	<.3§
O_1K_1 (Ewing)	13 (8- 20)	90	1.3 (1.1-1.6)	15 (9-17)
O_8K_{44} (")	6 (5- 10)	180	2.0 (1.6-2.4)	<.3
O_7K_1 (")	3 (1- 4)	400	1.9 (1.6-2.3)	10 (6-13)
O_2K_1 (")	0.4 (0.1- 3)	2700	1.7 (1.3-2.1)	14 (10-17)

* Relative to virulence of strain of serotype $O_{16}K$-.

† Avg and range of determinations on 2 to 5 batches of cells as determined by the Svennerholm procedure using N-acetylneuraminic acid as standard.

‡ Figures in parentheses are 95% confidence limits.

§ Small amounts of sialic acid have been reported for this strain(2).

minations on duplicate and triplicate hydrolysates of the same batch of cells gave sialic acid values which checked within 20% of each other. Samples were also checked by the Ehrlich(6) and the thiobarbituric acid (7) methods. Values for sialic acid found by using the Ehrlich test agreed within experimental error with the results of the resorcinol test. The thiobarbituric acid test was complicated, however, by the presence in some *E. coli* strains of material which gave no reaction in the resorcinol and Ehrlich tests, but which with thiobarbituric acid gave a chromophore with an absorption peak close to that produced by sialic acid.

The efficacy of the system was tested by recovery studies. Sialic acid (30 to 100 μg) was added to 10 to 100 mg batches of cells prior to hydrolysis. Recovery of added sialic acid was 90 to 100%. The lower limit of detection was 0.3 μg/mg cells. Thirty μg of sialic acid added to 100 mg of cells could readily be detected.

The presence of sialic acid in the bacteria was confirmed by paper chromatography. Concentrates of the column eluates from which the sodium was removed by passing them over a Dowex 50-X-8 (H+ form) resin column were chromatogrammed in 3 systems(8): 1-butanol:acetic acid:water (4:1:5); 2-butanol:acetic acid:water (4:1:5) and ethyl acetate:pyridine:acetic acid:water (5:5:1:3). The chromatograms were developed with a resorcinol spray(9). Samples‡ of N-acetylneuraminic acid and N-acetyl-4-O-acetylneuraminic acid served as standards and were run in parallel or mixed with the concentrates of the column eluates.

E. coli were disintegrated by shaking suspensions of cells (25 mg/ml) in water with powdered glass in a vial clamped on a shaker head (International Equipment Co. No. 6007) run at 1400 rpm for 20 minutes in an International centrifuge. The supernatant of the shaken suspension was filtered through (U.F.) sintered glass filters. The ability of these cell-free lysates to cause hemorrhagic lesions was tested by injecting aliquots combined with epinephrine (100 μg) in total volumes of 0.2 ml intradermally in the shaved abdomen of rabbits according to the procedure described by Thomas(10).

Results. In Table I the *E. coli* strains are listed in order of increasing lethality as determined by their LD_{50} values. For convenience, the strain of serotype $O_{16}K$-, which was the least lethal, was used as standard and the lethality of the other strains was expressed in relation to this strain. Using this

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‡ A sample of N-acetylneuraminic acid was kindly provided by Dr. R. J. Winzler (Univ. of Illinois) and samples of diacetylneuraminic acid were kindly donated by Dr. Gunnar Blix (Uppsala Univ., Sweden).

TABLE II. Lethality and Sialic Acid Content of *E. coli* Strains Grown for 5 and 24 Hour Periods.

Serotype of strain and origin	Age of culture, hr	LD_{50} for mice, No. viable organisms/mouse	Sialic acid, μg/mg cells, avg and range
O_2K_1 (DeWitt)	5	.3 (.1- .6) $\times 10^5$	14 (10-17)
	24	.5 (.3- 1.0) $\times 10^5$	$<.3$
O_1K_1 (Ewing)	5	37 (7 -57) $\times 10^5$	15 (9-17)
	24	56 (37 -87) $\times 10^5$	$<.3$
O_7K_1 (")	5	7 (4 - 9) $\times 10^5$	10 (6-13)
	24	13 (9 -18) $\times 10^5$	$<.3$

scale, for instance, the strain of serotype $O_{111}B_4$ is as lethal as the $O_{16}K$- strain, but the strains of serotype $O_{26}B_6$, O_1K_1 and O_2K_1 respectively are 12, 90 and 2700 fold more lethal.

The difference in lethality between strains is not accounted for by differences in heat stable toxins in the cells. LD_{50} values of the heat killed cells of the various strains did not differ significantly, with the possible exception of the strain of serotype $O_{16}K_1$ which appeared to be slightly less toxic than the other strains. (Table I).

The difference in lethality between strains is not accounted for by differences in sialic acid content. Three of the 5 most lethal strains, those of serotype O_2K_1, O_7K_1 and O_1K_1 were found to contain from 1 to 1.5% sialic acid (Table I). No sialic acid was found in the strain of serotype O_8K_{44}. Similarly no sialic acid was detected in hydrolysates of 200 to 300 mg of cells of the strain of serotype $O_{10}K_5$ although this strain has been reported(4) to contain small amounts of the acid. On the other hand the strain of $O_{16}K_1$ which is relatively avirulent contained as much sialic acid as each of the 3 more lethal strains. Paper chromatograms of hydrolysates of all 4 strains with the K_1 antigen showed the presence of resorcinol positive material which in 3 different systems could not be separated from a sample of authentic N-acetylneuraminic acid. No spots corresponding to N,O-diacetylneuraminic acid were observed; this substance, however, is known to be somewhat unstable and may degrade to the N-acetyl derivative(3). Our finding of sialic acid in strains containing the K_1 antigen is in agreement with observations by Barry(2).

During further studies we noted that the sialic acid content of cells grown in trypticase soy broth gradually decreased as the cells became older, to the point that in cells from cultures from 18 to 24 hours old none could be detected. This loss of sialic acid in the older cells was not permanent, for when these cells were used as inoculum in fresh broth, they proliferated and produced offspring containing sialic acid. The observation that older cells in contrast to younger cells contained little, if any, sialic acid offered the opportunity to compare the effect on lethality of the presence or absence of sialic acid in cells of the same strain. Differences in survival of the inocula in the host would be expected to be reflected in differences in lethality. Comparison of the lethality of 5 and 24 hour old cultures of strains of serotype O_2K_1, O_7K_1 and O_1K_1 are shown in Table II. Titrations yielding the LD_{50} values shown in Table II were performed about 12 months after the data shown in Table I were obtained. It can be seen by comparing the LD_{50} values for the 5 hour cultures of the 3 strains both in Table I and Table II that their lethality remained relatively constant. The 24 hour old cells of all 3 strains are only slightly less lethal than the corresponding 5 hour old cells (Table II); yet they contain, if any, only a fraction of the sialic acid present in the 5 hour old cells. No sialic acid was detected in hydrolysates made by exposing 100 mg of cells from 24 hour cultures to 0.1 N or 1 N H_2SO_4 at 80°C for one hour. In contrast, sialic acid was readily detected and measured in hydrolysates of as little as 15 mg of 5 hour cells of the strains of serotype O_1K_1, O_2K_1 and O_7K_1.

Cell-free lysates of 5 hour cells of the strain of serotype O_2K_1 contained sialic acid in bound form. This sialic acid could be released by mild hydrolysis, as verified by the thiobarbituric acid test which measures only free sialic acid, and as confirmed by paper chromatography. Aliquots of such lysates, containing 30 to 50 μg of N-acetylneuraminic acid in bound form, injected intraperitoneally in mice caused toxic symptoms including anorexia, loss of weight, lethargy and occasionally death. However, comparable lysates of 24 hour old cells of the same strain, which contained no sialic acid, caused the same symptoms when injected in mice. Further when injected intradermally in combination with epinephrine in the shaved abdomen of rabbits, both cell-free preparations produced hemorrhagic lesions at sites of the injections. These results indicated that sialic acid played no essential role in the toxicity of these cell extracts.

Discussion. The data tabulated in Table I show that there is no correlation between sialic acid content of cells of strains of *E. coli* and lethality for mice. Increased lethality must depend on survival of a small inoculum in the host and it is during the lag phase that the inoculum would be expected to be most vulnerable to host defense mechanisms. The 24 hour old cells which contained little, if any, sialic acid were as lethal and hence as able to survive in the host as the 5 hour cells which contained as much as 1 to 1.5% sialic acid (Table II). Hence, sialic acid does not play an essential role in protecting the cells during their lag phase from the host defense mechanism.

Summary. The lethality of 13 *E. coli* strains, each of a different serological type, was compared in mice and was found to differ greatly. These differences could not be attributed to differences in heat stable toxins nor to presence or absence of sialic acid in the cells. *E. coli* strains containing sialic acid gradually lost most, if not all, of this monosaccharide after 24 hours growth in culture, but no corresponding decrease in lethality occurred. Cell-free lysates of *E. coli* containing bound sialic acid were toxic for mice, and when injected in combination with epinephrine produced hemorrhagic lesions in rabbits, but no more so than did comparable lysates from older cells of the same strain which contained no sialic acid.

The technical assistance of Mrs. Helen N. Dilts is gratefully acknowledged.

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Dietary Composition and Tissue Protein Synthesis I. Effect of Tryptophan Deficiency.* (26838)

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Protein deficiency is a well known factor in growth retardation. A low dietary level of an adequate protein or of one of the essential amino acids in an otherwise complete diet is the most common way of inducing a protein deficiency. Protein free diets or com-

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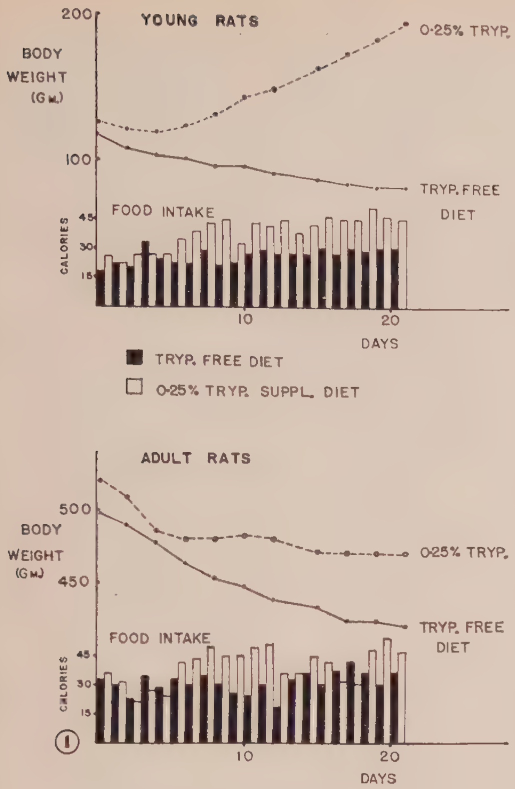

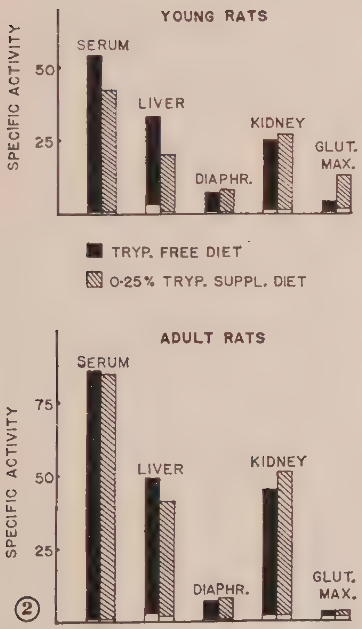

FIG. 1. Body wt changes and food intake of young and adult male rats fed a tryptophan-free diet and a diet supplemented with 0.25% tryptophan.

FIG. 2. Specific activities of tissue proteins of rats fed a tryptophan-free and a supplemented diet. Rats sacrificed on 13th day of experiment, 5 hr after inj. of 1-C^{14}-glycine. White area below each column represents non-protein fraction specific activity.

plete absence of an essential amino acid are the extreme experimental conditions often encountered. The loss of body and organ weight under these circumstances is well established as is the fact that not all organs are depleted at the same rate(1). It was our purpose to study different tissues as to their ability to synthesize protein as measured by uptake of injected C^{14}-glycine. Of special interest was the metabolism of skin and newly forming connective tissue such as that which is stimulated by subcutaneous implantation of plastic surgical sponge.

Methods. Young and adult male rats of the Holtzman strain were implanted subcutaneously with sterile polyvinyl sponges (Ivalon) and placed on tryptophan free 24% acid casein hydrolysate† and tryptophan supplemented diets(2). On the 13th day of experiment all rats were injected intramuscularly with 1-C^{14}-glycine (4.5 mc/mM) in saline solution. The adult rats received 1 μc (7.3×10^5 counts per minute) per 100 g of body weight and the young animals 3 μc (2.19×10^6 counts per minute) per 100 g of body weight. Because of the different levels of radioactivity injected relative values rather than absolute values should be compared among the different age groups.

One group of animals was sacrificed on the 13th day, 5 hours after receiving the labeled glycine. The other group was sacrificed on the 21st day of experiment, *i.e.*, 8 days following isotope administration. Sacrifice was performed by overexposure of the animals to ether. Skin, sponge, and the tissues under study were removed and protein and non-protein fractions and collagen isolated and counted as previously described(3). Collagen was determined by a modification of the Neuman and Logan method for hydroxyproline(4) after the samples had been hydrolyzed in 6 N HCl at 25 p.s.i. during 3 hours.

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† Salt Free Hydrolysed Casein, Sheffield Chemical, Division of National Dairy Products Corp., Norwich, N. Y.

All values for tissue protein radioactivity are expressed as specific activity (counts/minute/mg tissue protein or collagen/μc glycine-1-C^{14}).

Results. Changes in body weight of the different experimental groups are shown in Fig. 1. Young animals fed the tryptophan supplemented diet gained an average of 3.3 g per day. The non-supplemented group lost weight.

Neither diet when fed to adult rats was able to support body weight, although the tryptophan supplemented diet was more efficient in counteracting weight loss.

Collagen contents of skin and subcutaneously implanted polyvinyl sponge and percent of dry fat-free solids of the skin of the young animals 13 and 21 days after initiation of the experiment are shown in Table I. Values for dry fat-free skin solids were greater in the deficient animals than in the supplemented group at both the 13th and 21st day of experiment. A similar increase in skin collagen is also evident in the deficient group as compared to the supplemented control. This does not mean increased synthesis but probably a change in the relative composition of skin. However, sponge collagen was significantly increased in those animals fed the complete diets, thus indicating that formation of new connective tissue proceeds at a faster rate during the feeding of an adequate diet.

The specific activity of collagen isolated from skin and sponge of these animals was also determined. These values were meant to furnish an idea of the rate of collagen synthesis at the different sites.

Thus the rate of collagen synthesis in the skin, as judged by specific activity, occurring in the tryptophan supplemented rat is 4 times that observed in the unsupplemented animal. In direct contrast to the skin, no significant differences are to be observed in the newly formed sponge collagen. The slightly decreased specific activity of sponge collagen in Group IV could be accounted for by the larger amount of collagen present at the end of the 21st day, causing an isotopic dilution which followed the peak of maximum glycine-C^{14} incorporation.

It seemed of interest to calculate the ratio of sponge to skin collagen specific activities. This ratio could be interpreted as an index of the synthesis of new collagen in response to the stress of a sponge implant as compared to its synthesis in the skin.

In the tryptophan supplemented animals

TABLE I. Total Content and Specific Activity of Skin and Subcutaneously Implanted Sponge Collagen of Young Growing Animals Fed a Supplemented and Unsupplemented Tryptophan-Free Diet for Varying Periods.

Group	Supplement	Exp. period (days)	% dry fat-free skin solids	Skin collagen (% wet wt)	Total sponge collagen synthesized (mg)	Time after isotope inj.	Collagen specific activity†		
							Skin	Sponge	Ratio, sp./skin
I	None	13	24.7	13.9 ± 1.1*	10.1 ± .8	5 hr	12 ± 2.3	56 ± 6	4.7
II	.25% try.	13	21.8	9.5 ± .7	13.2 ± .3	5 "	49 ± 11.0	60 ± 2	1.2
III	None	21	27.0	13.8 ± .5	15.2 ± .9	8 days	8.4 ± 1	58 ± 12	6.9
IV	.25% try.	21	22.7	11.2 ± .9	22.0 ± .9	8 "	23.3 ± 2	46 ± 6	2.0

* Stand. error.

† Counts/min./mg collagen μc glycine-1-C^{14}/100 g body wt.

the ratio was close to 1 (Group II) indicating that collagen synthesis proceeded at a constant rate throughout the body at that time. In Group IV isotope dilution would again account for a slight increase in ratio 8 days following tracer injection. This ratio was increased in the deficient rats indicating a sparing effect which would favor new tissue formation as a response to sponge implant during inadequate nutrition.

TABLE II. Total Content and Specific Activity of Skin and Subcutaneously Implanted Sponge Collagen of Old Rats Fed a Supplemented and Unsupplemented Tryptophan-Free Diet for Varying Periods.

Group	Supplement	Exp. period (days)	% dry fat-free skin solids	Skin collagen (% wet wt)	Total sponge collagen synthesized (mg)	Time after isotope inj.	Collagen specific activity: Skin	Collagen specific activity: Sponge	Collagen specific activity: Ratio, sp./skin
V	None	13	29.2	17.6 ± .5	11.6 ± 1.3	5 hr	7 ± .4	83 ± .9	11.8
VI	.25% try.	13	28.5	17.4 ± .5	12.6 ± .8	5 "	9 ± .9	100 ± 4.1	11.1
VII	None	21	30.9	17.9 ± .5	15.8 ± .7	8 days	8.3 ± 1	113 ± 10	13.6
VIII	.25% try.	21	28.7	16.8 ± .1	14.9 ± .9	8 "	9.3 ± .6	106 ± 11	11.4

Results of the experiments in older adult rats are shown in Table II. Tryptophan deficiency slightly increased the percentage of dry fat-free skin solids. Skin collagen values were not significantly altered by the nature of the diet. They were elevated in all groups with respect to the younger animals. Sponge collagen determinations failed to show significant differences in net synthesis between tryptophan supplemented and unsupplemented groups.

Table II summarizes specific activity determinations of the collagen samples isolated from adult rats. Skin as well as sponge collagen values in the tryptophan supplemented group were slightly higher after 5 hours post injection of C^{14}-glycine.

Because of the slow rate at which skin collagen is synthesized in the adult rat, the isotopic dilution effect was not observed in these animals. The sponge/skin ratio was almost constant. These results seem to indicate that in adult animals dietary imbalance does not cause the marked changes in rate of collagen synthesis that are observed in younger animals. However, body weight loss occurred in all adult rats regardless of presence or absence of tryptophan in the diet.

Fig. 2 summarizes results obtained when rate of labeling of different tissue proteins other than collagen were studied in young and adult rats receiving the tryptophan deficient and supplemented diets over a 13-day period. The specific activities of tissue proteins 5 hours after injection of 1-C^{14}-glycine are compared. Non-protein tissue radioactivity is represented by the white area below each column.

The marked differences in growth observed in young animals lacking tryptophan in the diet were not reflected in the rate of tissue protein labeling. Serum and liver proteins of the young deficient animals had a higher specific activity than that of the supplemented ones. On the other hand the gluteus muscle of the tryptophan supplemented rats incorporated the labeled glycine more efficiently.

The radioactivity of the non-protein moiety was increased in those organs where protein synthesis was enhanced. Liver and gluteus muscle showed the most significant increase of this fraction. This agrees with the findings of Clark(5) and our observations on the stimulating effect of testosterone propionate on different tissues in which an increased rate of protein synthesis was always accompanied by a concomitant increase in non-protein nitrogen or radioactivity of this fraction (3).

A similar pattern was observed in adult animals, though no increased labeling was apparent in the gluteus muscle.

Discussion. Both age and tryptophan deficiency seemed to increase the amount of fat-free solids in the skin. Also the relative amount of skin collagen present was increased by the dietary deficiency in growing animals. Adult rats all showed a greater collagen content than young rats, independent of the nature of the diet. These changes in collagen content should not be equated to a net synthesis but rather to a change in relative composition of skin. Fat and water content of the skin are markedly influenced by the nutritional state and can be responsible for changes in total protein content of the skin(6). Also the non-collagenous skin proteins are more readily depleted by nutritional deficiency than the collagen fibers, which have a much slower turnover rate. The specific activity of skin collagen isolated 5 hours after injection of C^{14}-glycine was 4 times greater in young animals receiving the complete diet than in the deficient controls. In spite of the lower relative amount of collagen present in skin the rate of synthesis was far more active in these animals. This agrees with the fact that the supplemented animals were growing whereas the deficient were losing weight.

Total collagen synthesized around the implanted sponge was greater in the young animals receiving tryptophan as a dietary supplement. No such difference was observed in older rats receiving the same supplement. A decreased collagen content of healing wounds and of subcutaneously implanted sponge biopsies of protein depleted rats has been demonstrated(7,8).

Nevertheless rate of synthesis of sponge collagen did not vary significantly with the nature of the diet as occurred with the skin. A ratio of sponge/skin specific activities was calculated, to serve as an index of how the organism favors collagen synthesis at different levels as a consequence of variables in diet and age. Older animals under all circumstances greatly favored synthesis of collagen at the sponge site. In these animals the diet had no apparent effect and did not influence the balance in rate of synthesis between sponge and skin.

The surprising fact that rate of uptake of labeled glycine by several tissues studied was not reduced by lack of tryptophan in the diet, but increased in serum proteins and liver suggests that at least at these sites protein synthesis occurs normally or, as seems apparent, at an increased rate. Similar findings have been reported by Sidransky and Farber(9) who found that rats fed a threonine deficient diet incorporated several radioactive amino acids into liver and serum proteins at a faster rate than rats fed supplemented diets. Waterlow(10) feeding rats a low protein diet found that protein-depleted animals incorporated S^{35}-methionine into the visceral organs (including liver) at a faster rate than the normal controls. In spite of this increased labeling during protein deficiency all animals consistently lost weight. The liver, where protein synthesis would seem to be most enhanced by nutritional deficiency is, however, one of the most rapidly depleted organs(1). This would suggest that increased protein synthesis occurs simultaneously with an even greater increased protein catabolism. The stimulation of protein synthesis could be the cellular response to the enhanced catabolic state.

The possibility that this mechanism is under hormonal control is being studied. The possibility that dietary deficiency (amino acid deficiency, amino acid imbalance, etc.) may cause increased mobilization of protein stores of the body should be considered as a likely cause of turnover stimulation at such sites as liver and serum.

Summary. Rate of collagen synthesis, as measured by 1-C^{14}-glycine uptake, by skin

and in subcutaneously implanted polyvinyl sponge has been shown to vary with age and diet. A tryptophan-deficient diet significantly reduced the rate of skin collagen synthesis in young animals. However, the greater loss of more labile components of the skin resulted in a relative greater percentage of skin collagen in this group. Rate of synthesis in the sponge was not significantly affected. The deficient animals showed an increased specific activity in liver and serum proteins, suggesting that protein synthesis probably occurs at a faster rate in the liver simultaneously with increased catabolism or utilization.

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Identification of 24-Dehydrocholesterol in Human Blood Vessels Following MER-29 Therapy.[*] (26839)

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Avigan *et al.*(1) have demonstrated that inhibition of sterol biosynthesis by MER-29 (Triparanol) results in accumulation of 24 dehydrocholesterol in rat livers. 24-dehydrocholesterol has been identified by Steinberg *et al.*(2) in the blood of humans given MER-29. Little is known of the biologic activity of 24-dehydrocholesterol in man except that it does not appear spontaneously in human blood(3). The use of MER-29 for treatment of hypercholesterolemia in man makes it important to learn if 24 dehydrocholesterol accumulates in blood vessel walls as well as in blood.

Materials and methods. Vessels were obtained at autopsy from a 16-year-old white male whose diagnoses were: familial hypercholesterolemia, congenital aortic stenosis and recent myocardial infarction. The patient had received 250 mg per day of MER-29 for 79 days. Extensive atheromatosis covered 90% of the area of the aortic wall. Specimens of normal vessel wall were taken from between atheromas and stripped of adventitia. Atheromas were graded(4), freed from surrounding tissue, and stripped of adventitia.

Serum was saponified and extracted with petroleum ether as described by Abell *et al.* (5). Tissue specimens were saponified in 20% ethanolic KOH and extracted with one volume of water and 2 volumes of petroleum ether. Petroleum ether residues were taken up in chloroform for gas liquid chromatography (GLC).

GLC was performed in ⅛ inch glass columns, packed with Gas Chrom P[†] coated with neopentyl adipate terminated (NAT), neopentyl glycol sebacate (NGS) as described by Lipsky(6), or QF-1 (10,000 C.S.) as described by VandenHeuvel *et al.*(7). An argon ionization detector containing radium was used and was calibrated with a known mixture of cholesterol and 24-dehydrocholes-

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TABLE I. Appearance Time of Peak Concentration, Liver, and Normal Aortic Wall Sterols.

Charge		Appearance time (min.)		
		NAT*	QF-1†	NGS‡
Liver sterols (2 peaks)	1.	13	70	16.3
	2.	17	88	20.0
Aortic sterols (2 peaks)	1.	13	70	16.3
	2.	17	88	20.0
Cholesterol		13	70	16.3
24-dehydrocholesterol		17	88	20.0

* 4 ft ⅛ inch column, 3% NAT, 231°C, 300 ml/min. flow.

† 6 ft ⅛ inch column, 1% QF-1, 217°C, 300 ml/min.

‡ 3 ft ⅛ inch column, 1.5% NGS, 231°C, 400 ml/min.

terol.[‡] Relative peak areas of cholesterol and 24-dehydrocholesterol in unknown mixtures were measured, corrected in proportion to peak areas of the standard mixture, and 24-dehydrocholesterol was expressed as a percent of cholesterol plus 24-dehydrocholesterol.

Serum sterols were also assayed by the method of Abell *et al.*(5), modified as follows: Color developed was read after 30 min at 620 mμ; and 90 min at 420 mμ. Apparent desmosterol and cholesterol were calculated from the data of Avigan *et al.*(8) and Frantz *et al.*(9).

Results. During a 3-month period prior to MER-29 therapy total serum sterols estimated colorimetrically ranged from 390 to 525 mg% (6 determinations) and there was no evidence of 24-dehydrocholesterol. During 79 days of MER-29 therapy total serum sterols estimated colorimetrically ranged from 407 to 512 mg % (6 determinations). During the last 40 days of MER-29 therapy, 24-dehydrocholesterol could be demonstrated in serum. By colorimetric determination, 24 dehydrocholesterol was estimated to be 19-24% of total serum sterols; separation by GLC indicated that 24-dehydrocholesterol comprised 25 to 32% of total serum sterols.

The day preceding death, total serum sterols were 407 mg% and 24 dehydrocholesterol was 32% of total (determined by GLC). Sterols from liver and normal blood vessel wall removed at autopsy were separated by GLC and 2 peaks were found. These peaks had appearance times identical with that of cholesterol and 24-dehydrocholesterol when chromatographed on columns of NAT, NGS, and QF-1 (Table I).

In 3 liver specimens, 24-dehydrocholesterol was 23-29% of total sterol. In normal aorta, from 3 locations, 24-dehydrocholesterol was 13-16% of total sterol. In normal iliac arterial wall, 24-dehydrocholesterol was 8.8% of total sterol. Ten atheromas (4 grade II, 6 grade III) demonstrated no evidence of 24 dehydrocholesterol.

Summary and conclusions. This report indicates that 24-dehydrocholesterol accumulated in human blood vessel walls and it seems probable that treatment with MER-29 was responsible. Although 24-dehydrocholesterol was not demonstrable in the extensive atheromatosis which covered 90% of the aorta, it accounted for 13-16% of the sterols in normal aortic wall. The magnitude of accumulation of 24-dehydrocholesterol in normal aortic wall would not have been apparent if assays had been performed on sterols extracted from the aorta as a whole because the sterols of normal wall would have been greatly diluted by atheromatosis lipid containing no 24-dehydrocholesterol. Because these vessels were examined only 79 days after onset of therapy with MER-29, the presence of 24-dehydrocholesterol in normal wall but not in atheromas seems best explained by a more rapid accumulation in normal wall. However, it is possible that 24-dehydrocholesterol may only accumulate in normal vessel walls.

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Assay of Serum Vitamin B_{12} Concentration Using Co^{57}-B_{12} and Intrinsic Factor. (26840)

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Athough many proteins are capable of binding Vit. B_{12}, the greatest avidity for the vitamin is exhibited by intrinsic factor (I.F.). Barlow and Frederick(1) reported that purified intrinsic factor concentrates reveal several distinct protein components on paper electrophoresis but the most intense binding of Co^{60}-labeled Vit. B_{12} was observed in the region with the least amount of stainable protein material.

Bunge and coworkers(2) have clearly shown that Co^{60}-B_{12} (B_{12}*) and unlabeled Vit. B_{12} are biologically identical and compete for the binding sites of normal human gastric juice.

In agreement with observations of other investigators(1) initial studies in this laboratory using paper electrophoresis demonstrated that in mixtures of I.F. and B_{12}*, where there was insufficient I.F. to bind all the B_{12}*, 2 peaks of radioactivity could be demonstrated; the free B_{12}* remaining close to the origin and the B_{12}*-I.F. complex moving toward the anode. If concentrations of I.F. and B_{12}* were kept constant while concentration of unlabeled Vit. B_{12} was varied, the ratio of bound B_{12}* to free B_{12}* (B/F) decreased progressively with increase in concentration of unlabeled B_{12} in consequence of the competitive inhibition of binding of the labeled vitamin.

These results are analogous to the observations of Berson, Yalow *et al.*(3) on binding of insulin-I^{131} to insulin binding antibodies, in studies employing paper electrophoresis and chromatography. The observed inverse relationship between the ratio of bound to free I^{131}-insulin (B/F) and total concentration of insulin present was exploited in development of an immunoassay for insulin in plasma(4).

In the present communication the application of these principles to assay of Vit. B_{12} in plasma is described.

Methods. Since the specific activity of commercially available labeled B_{12} is at present too low to yield adequate counting rates in small amounts of serum extracts containing tracer quantities of B_{12}*, electrophoretic separation of free and bound B_{12}* proved inexpedient. Therefore, attempts were made to separate bound and free Vit. B_{12}* by precipitation of I.F.-B_{12}* complexes from larger quantities of serum extract than could be conveniently analyzed by paper electrophoresis. Several protein precipitants (TCA, phosphotungstic acid, etc.) produced co-precipitation of free Vit. B_{12} and could not be used for the present purpose. The Somogyi, barium hydroxide-zinc sulfate method(5) proved satisfactory.

Because of its higher available specific activity, Co^{57}-B_{12} (5 μc/μg)† was used instead of the Co^{60}-labeled vitamin.

Preparation of plasma extracts. Most or all of the Vit. B_{12} in serum is present as a heat-labile Vit. B_{12}-protein complex. When serum is acidified and heated the proteins are precipitated and Vit. B_{12} is liberated. Accordingly, Vit. B_{12} extracts were prepared by diluting one ml of the test serum with 2 ml of distilled water and 1 ml of 0.1 N hydrochloric acid. After agitation at 100°C for 30 minutes, the precipitated proteins were separated by centrifugation and one ml of the supernatant (representing 0.25 ml of the original serum) was assayed for Vit. B_{12} content. Recovery by this extraction procedure of 100 $\mu\mu$g of Co^{57}-B_{12} added to serum was 85-88%. Recovery of added unlabeled Vit. B_{12} is reported below.

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† Purchased from Merck, Sharp & Dohme, Rahway, N. J.

Preparation of standard solutions. Since the binding of Vit. B_{12} to intrinsic factor may be influenced by such factors as pH, and concentrations of various electrolytes notably calcium(6), it is essential that a "standard curve" be obtained under conditions which simulate as closely as possible those obtained in the presence of unknown samples. Since these conditions have not yet been completely determined, standard solutions, containing known amounts of Vit. B_{12} have been prepared using an extract of serum from a Vit. B_{12} deficient patient. Fig. 1 illustrates the different curves obtained with the same amount of Co^{57}-B_{12} and I.F. added to unlabeled B_{12} in distilled water and B_{12} deficient serum extract.

The serum extract employed for preparation of the standard solution used in the present study (serum C) contained the lowest concentration of Vit. B_{12} yet encountered as evidenced by the least inhibition of binding of Co^{57}-B_{12}. Intrinsic factor‡ was progressively diluted in this extract until a quantity was reached (0.00035 mg) in which 65%-75% of 100 $\mu\mu$g Co^{57}-B_{12} was bound under conditions to be described. A series of 1 ml mixtures was then prepared in which concentration of unlabeled crystalline Vit B_{12} ranged from 0-250 $\mu\mu$g/ml but in which concentrations of Co^{57}-B_{12} (100 $\mu\mu$g/ml) and I.F. (added last in all cases) were maintained constant. After one hour incubation at room temperature with gentle agitation, 1.5 mg of human serum albumin was added to all tubes in negligible volume (6 μl) to serve as carrier protein in the precipitation. This was followed by addition of 0.5 ml of 5% $ZnSo_4$. $Ba(OH)_2$ was then added dropwise with agitation after each drop until the endpoint of $ZnSo_4$ precipitation was reached. Amount of $Ba(OH)_2$ used was previously determined by titration with phenolphthalein indicator(5). The filtrate, representing free Vit. B_{12}, was assayed for radioactivity in a well type scintillation counter, using a gamma ray spectrometer with a sensitivity of 1.6×10^6 CPM/μc Co^{57}-B_{12}. Each sample was counted for a minimum of 10,000 counts.

In the absence of I.F. and serum albumin no Co^{57}-B_{12} was precipitated. In the absence of I.F. but with serum albumin present at the concentrations noted considerably less than 5% Co^{57}-B_{12} was precipitated.

For determination of Vit. B_{12} in unknown samples, test serum was prepared as described, and 1.0 ml of the extract was substituted for the unlabeled Vit. B_{12} used in the standard solution.

Vit. B_{12} concentrations were determined in the sera of 8 normal subjects, 8 patients with megaloblastic anemia, 1 patient with acute monocytic leukemia and 1 patient with chronic myelogenous leukemia.

Results. The ratio of bound Co^{57}-B_{12} to free Co^{57}-B_{12} (B/F) is plotted as a function of unlabeled Vit. B_{12} concentration added to serum extract C to yield a standard curve (Fig. 2). Serum C was then assayed against the standard curve and proved to fall out on the zero point. Vit. B_{12} concentrations in extracts from unknown serum samples were determined from the B/F ratios obtained with these extracts by reference to the standard curve, as indicated in Fig. 2. Results are given in Table 1. Samples A through H were from patients with megaloblastic anemias, presumed to be deficient in B_{12}. Range of B_{12} levels is zero to 72 $\mu\mu$g per ml of serum. Samples I through P are normal sera in which Vit. B_{12} concentrations ranged from 104 to 328 $\mu\mu$g per ml.

Recovery of crystalline Vit. B_{12} added to serum before extraction is summarized in Table II. Correction for 15% loss in extraction has been made to permit estimate of the accuracy of the assay procedure. Range of

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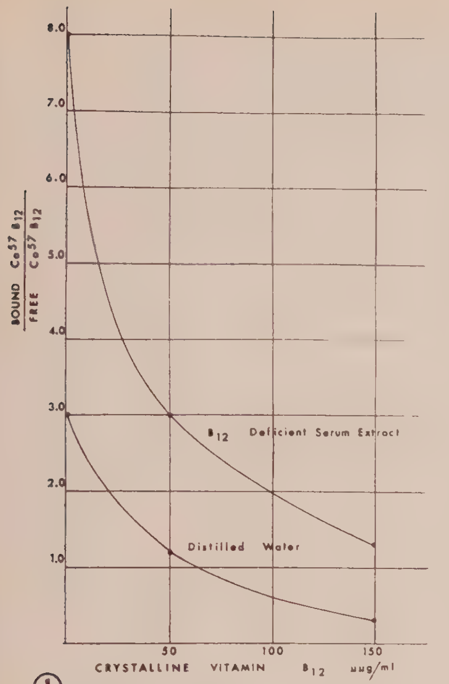
‡ Hog Intrinsic Factor concentration kindly supplied by Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N. Y.



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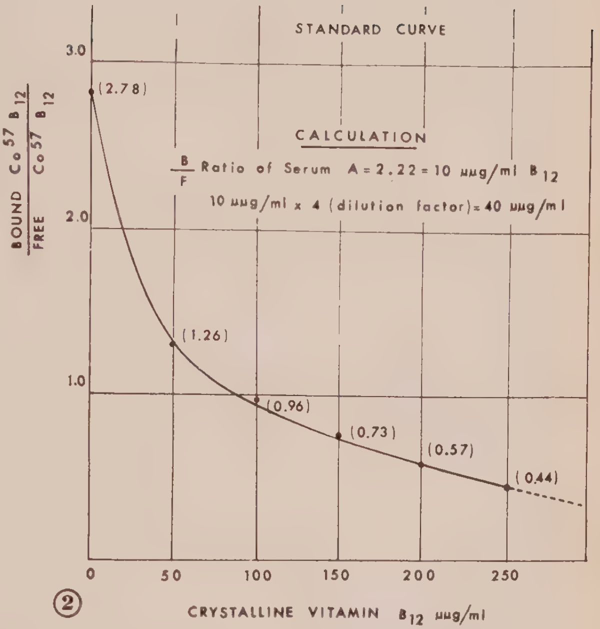

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FIG. 1. Different curves obtained using the same amount of I.F. and $Co^{57}B_{12}$ in distilled water and B_{12} deficient serum extract.

FIG. 2. Standard curve obtained by addition of crystalline Vit. B_{12} to extract of serum "C."

recovery was from 83 to 112%.

Since it is known that leukemic patients have a high serum B_{12} concentration, 2 such sera were assayed. Both had levels well over the normal range. The serum of the patient with acute monocytic leukemia showed a B/F value at the end of the curve, representing 1000 μμg per ml. The value for the serum of the patient with chronic myelogenous leukemia fell off the curve, yielding, by extrapolation, an estimate of 1500 to 2000 μμg per ml.

Discussion. The method described depends on an accurately assayed commercial preparation of crystalline Vit. B_{12}, since it is used in preparation of the standard curve. Variations in the preparations of I.F. or the presence of non-specific binders should not affect this method because the quantity used is constant for both standard solutions and unknown samples.

The values obtained by this method, as presently described, do not include the endo-

TABLE I. Serum Vitamin B_{12} Concentration in Patients with Megaloblastic Anemia, Leukemia, and Normal Controls.

Serum	Clinical condition	% free Co^{57}-B_{12}	Co^{57}-B_{12}, Bound / Free	Vit. B_{12} conc., μμg/ml
A	Megaloblastic anemia	31.0	2.22	40
B	"	28.8	2.47	20
C*	"	25.7	2.90	0
D	"	31.0	2.22	40
E	"	31.0	2.22	40
F	"	33.6	1.98	68
G	"	32.6	2.06	56
H	"	34.0	1.94	72
I	Normal	43.6	1.29	200
J	"	37.0	1.70	104
K	"	45.3	1.20	224
L	"	48.4	1.06	304
M	"	45.2	1.20	224
N	"	42.2	1.37	176
O	"	49.6	1.02	328
P	"	46.4	1.15	248
AL	Acute leukemia	69.5	.44	1000
CL	Chronic "	81.4	.23	1500-2000

* This serum extract used as standard.

TABLE II. Total Recovery of Crystalline Vitamin B_{12} Added to Serum before Extraction. All values in $\mu\mu g/ml$.

Serum (Table I)	Endogenous Vit. B_{12} level	B_{12} added to serum before extraction	Expected additional B_{12} in extract*	Vit. B_{12} level assayed	% recovery of expected total
E	40	100	85	104	83
F	64	150	127	192	100
G	56	200	170	188	83
J	104	100	85	236	112
K	224	150	127	312	89
L	304	200	170	504	106
M	224	300	255	408	85

* Correction for 15% loss secondary to extraction procedure.

genous B_{12} level of the serum extract used in preparation of the standard curve. However, this error is minimized by choosing a serum with the lowest B_{12} level (least inhibition of binding of 100 $\mu\mu g$ Co^{57}-B_{12} by I.F.).

Serum Vit. B_{12} concentrations in normal controls and in patients with pernicious anemia and leukemia reported above agree, in general, with levels obtained employing microbiological assay procedures(7,8). A more precise and critical comparison can be made only when many more sera are assayed simultaneously by both methods.

Summary. Based on some observed properties of the B_{12}-I.F. complex, an assay procedure is described for determining serum Vit. B_{12} levels using isotopically labeled Vit. B_{12} and intrinsic factor. 83%-112% of crystalline Vit. B_{12} added to serum was recovered in the assay. Concentrations of Vit. B_{12} in normal, B_{12} deficient, and leukemic sera were in the ranges, 104-328 $\mu\mu g$/ml, 0-72 $\mu\mu g$/ml and over 1000 $\mu\mu g$/ml respectively.

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Antigen Elimination from the Site of Arthus Phenomenon in the Guinea Pig* (26841)

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Almost 60 years have passed since Arthus (1), using horse serum injected into rabbits, described the phenomenon that bears his name, but its effect on the removal of antigen from the injection site has not been clearly delineated. The gross physical manifestations of this reaction are usually apparent 60 minutes after injection of the antigen, and thus one might anticipate an effect on antigen removal during the first few hours after in-

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jection. We have not found data in the literature comparing rate of removal of soluble antigen during the first 12 hours after subcutaneous injection into immune and control animals. Wadsworth(2) in 1904, showed that when virulent pneumococci were placed in the lungs of immune rabbits, the degree of septicemia was less than in controls. Immune animals would develop diffuse exudative lesions comparable to the lobar pneumonia of man, but they ordinarily survived, whereas control animals died with bacteremia.

Opie(3) demonstrated that more egg albumin remained at injection site at 24-72 hr in immune rabbits than in control animals. He did not make observations at less than 24 hours and used large amounts of antigen (25 mg).

Krause(4), studying tuberculous infection in the guinea pig, observed that in the immune animals. after virulent subcutaneous infection a great delay occurs in transmission of tubercle bacilli from the point of entry to the customary places of localization within the body; he attributed this delay to the mechanical effect of the inflammatory process.

Menkin's(5) studies showing slower removal of india ink or bacteria from the nonspecifically inflamed peritoneal cavity are not necessarily analogous to the effects of inflammation due to immune processes.

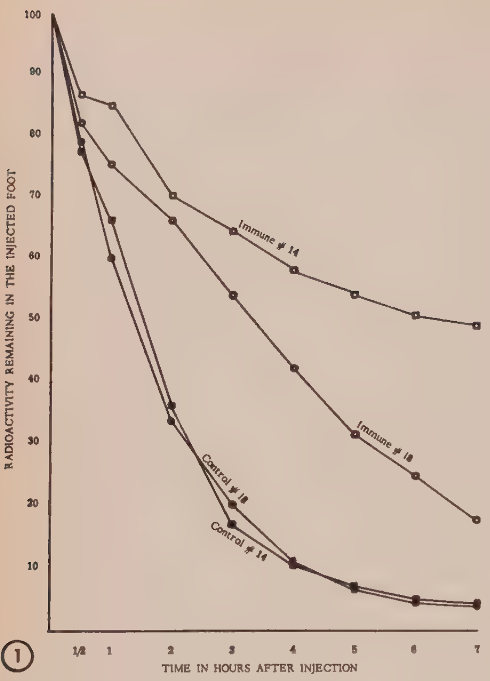
Rich(6), demonstrated that pneumococci are localized at site of intracutaneous injection in immune animals. He interpreted his data as showing that antibodies acted specifically to immobilize bacteria before the inflammatory response was apparent.

Recently, Talmage, Dixon, Bukantz and Dammin(7), and Law and Wright(8), studying the rate of antigen elimination after intravenous injection, noted a more rapid fall in blood antigen concentration in immune animals compared to normals.

In the following experiments, with the aid of radioisotopes, we have studied the effect of the Arthus phenomenon on elimination of the antigen from the subcutaneous tissue of immune and control guinea pigs. We have also observed the effect of such an immune response on rate of disappearance of non-antigen from the site of the Arthus phenomenon.

Materials and methods. A. Materials: 1. Young adult guinea pigs (280-300 g, about 4 weeks old) were fed Purina chow *ad libitum*. Immune and control animal pairs carry the same number for identification. 2. Armour crystallized bovine serum albumin, (BSA). 3. Human gamma-globulin (HGG), isolated from a myeloma patient(9). 4. Cyanocobalamin with Co^{60} incorporated biosynthetically ($B_{12}Co^{60}$). 5. Human plasma albumin (HPA). 6. Freund's adjuvant (Bayol 8.5, Arlacel 1.5, and 5 mg/ml of mycobacterium bovis). *B. Methods*: 1. For immunization each guinea pig received an emulsion of 2.5 mg crystallized bovine serum albumin in 0.25 ml saline and 0.25 ml Freund's adjuvant. Three weeks later sera from heart blood were tested qualitatively for precipitating antibodies; the test was positive in 22 out of the 26 immunized guinea pigs. *This was the only immunizing antigen* injected and throughout this report "immune animal" refers to one given BSA in Freund's adjuvant 3-5 weeks before these studies. 2. BSA and HGG were radioiodinated according to the technic of Talmage *et al.*(7); at least 95% of I^{131} was in trichloracetic acid precipitate. 3) The radioactive solution to be studied (0.1 ml) was injected from a tuberculin syringe into the pad of a rear foot. *Specific details concerning amount and kind of material injected for study are found in the legends for tables and figures.* 4) Radioactivity remaining in the injected foot was assayed *in vitro* or *in vivo* with a scintillation detector coupled to a single channel pulse height analyzer. When 2 different isotopes (I^{131} and Co^{60}) were injected simultaneously the amount of each remaining was measured by scintillation spectrometry.

Results. Swelling, redness and edema and increased heat were noted during the first hour in the antigen injected foot of immune animals having precipitins in their serum. The Arthus reaction was lacking or minimal in those guinea pigs which did not develop precipitins. I. Removal of antigen from the site of injection in immune and control guinea pigs: a) Fig. 1, 2 and 3 show radioac-



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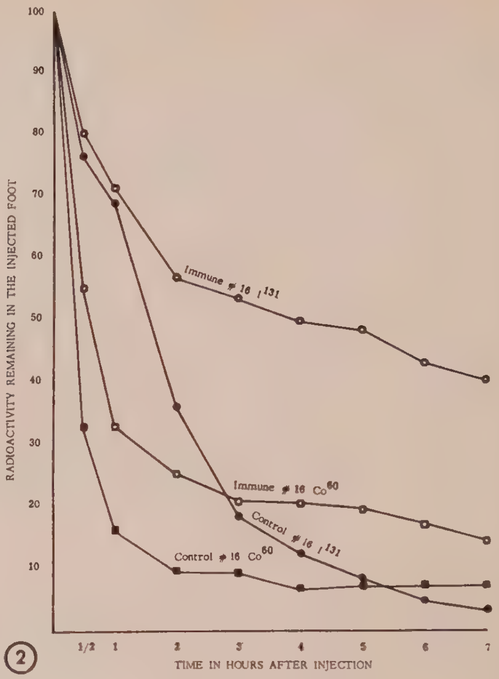

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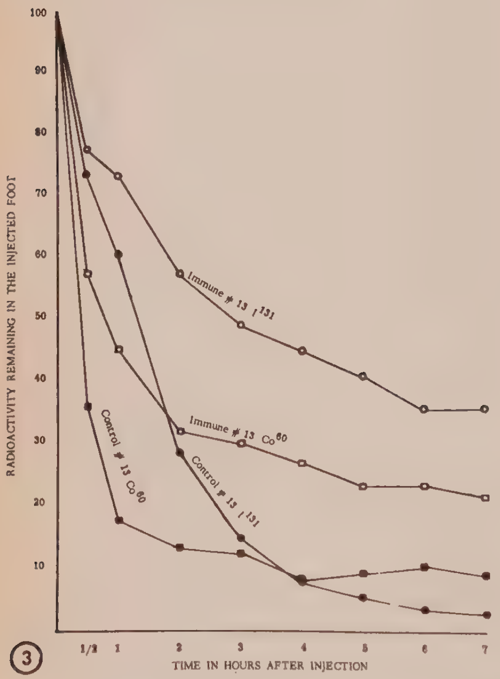

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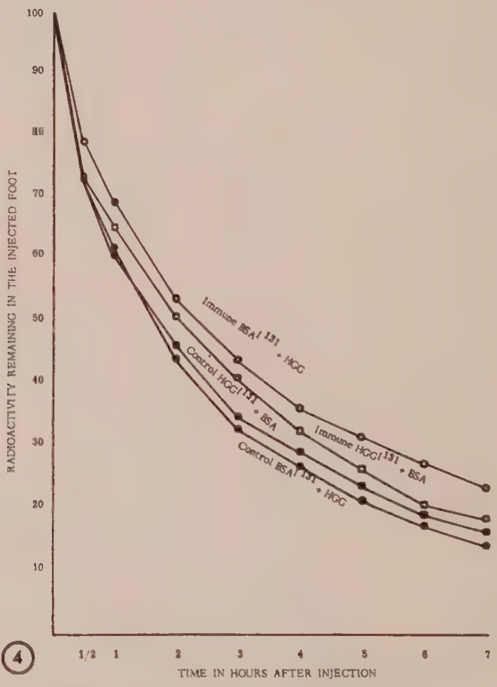

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FIG. 1. Two immune and 2 control guinea pigs inj. with 0.6 mg of BSA I^{131} in right rear foot. Each line represents *in vivo* determination of radioactivity remaining in inj. foot in one animal.

FIG. 2. One immune and one control guinea pig inj. in right rear foot with a mixture of 0.6 mg of BSA I^{131} and 0.5 μg of Vit. $B_{12}Co^{60}$, the latter being in excess of B_{12} binding capacity of the protein. Each line represents *in vivo* determination of radioactivity due to I^{131} or Co^{60} remain-

TABLE I. Comparison of Amount of Antigen Remaining at Injection Site in Immune and Control Animals.

Animal pair No.	Hr after inj.	Right hind foot inj. with	Radioactivity (I^{131}) remaining in right hind foot (% of inj. dose)	
			Immune guinea pigs	Control guinea pigs
6	.5	$BSA\,I^{131}$	84	42
7	.5	” + $B_{12}Co^{60}$	82	75
3	1	”	92	31
8	1	” + $B_{12}Co^{60}$	69	77
4	2	”	51	58
9	2	” + $B_{12}Co^{60}$	53	75
5	4	”	32	15
10	4	” + $B_{12}Co^{60}$	28	19
18	7	”	20	2
14	7	”	66	4
13	7	” + $B_{12}Co^{60}$	48	3
16	7	” + $B_{12}Co^{60}$	52	4
15	7	” + HPA	34	8
17	7	” + HPA	22	3
19	7	” + HGG	36	12
20*	7	” + HGG	25	14
21*	7	” + HGG	14	16
22*	7	” + HGG	20	19
23*	7	” + HGG	28	14
24	7	” + HGG	17	14
25	7	” + HGG	22	8
26	7	” + HGG	31	9
27	7	” + HGG	20	12
11	8	” + $B_{12}Co^{60}$	19	5
1	24	”	20	1
12	24	” + $B_{12}Co^{60}$	2	1

Radioactive antigen remaining in inj. foot of all immune and control guinea pigs. After sacrifice of animal right hind foot was amputated and radioactivity determined in a well scintillation counter. Animals inj. in right foot with $BSA\,I^{131}$ + HPA or $BSA\,I^{131}$ + HGG were inj. in left foot with $HPA\,I^{131}$ + BSA or $HGG\,I^{131}$ + BSA respectively. Dose of BSA, HPA and HGG = 0.6 mg of each in each inj. foot. Dose of $B_{12}Co^{60}$ = 0.05 μg.

* Immune animals with negative precipitin test.

tivity (as determined by *in vivo* counting) remaining in the foot at intervals up to 7 hr after injection of radioactive antigen. They demonstrate a marked slowing of removal of the antigen from the foot of the immune animals. The same trend is seen in Fig. 4 which shows mean radioactivity due to antigen in the injected foot of 9 immune and 9 control guinea pigs injected with a mixture of BSA and HGG.

b) Table I records the remaining radioactivity of $BSA\,I^{131}$ counted *in vitro* in the injected foot of all of animals used in our experiments. Here, there is also a significantly greater retention of antigen in the foot of the immune animals. The discrepancy seen in 4 control animals, No. 8, 4, 9, 21, probably represents variable tissue sites of injection. The results of these *in vitro* radioactivity determinations are analogous to those observed *in vivo* before sacrificing the animals. We conclude that antigen disappears from the site of subcutaneous injection at a slower rate in the immune than in the non-immune guinea pig.

II. Effect of Arthus phenomenon on disappearance of non-antigen from injection site: a) Effect on the water soluble small molecular weight compound (Vit. $B_{12}Co^{60}$). Fig. 2 and 3 and Table II show that in the presence of an Arthus phenomenon there is a slowing in absorption of Vit. $B_{12}Co^{60}$ but this effect is not noted during the first hour after

TABLE II. Comparison of Amount of Non-antigen (Vit. $B_{12}Co^{60}$) Remaining in Control Foot and Foot with Arthus Phenomenon.

Animal pair No.	Hr after inj.	Right hind foot inj. with	Radioactivity (Co^{60}) remaining in right hind foot (% of inj. dose)	
			Immune guinea pigs	Control guinea pigs
7	.5	$BSA\,I^{131}$ + $B_{12}Co^{60}$	49	55
8	1	*Idem*	48	51
9	2	”	31	25
10	4	”	18	8
16	7	”	13	2
13	7	”	22	5
11	8	”	7	3
12	24	”	14	9

Radioactivity of Vit. $B_{12}CO^{60}$ remaining in inj. foot. The difference between immune and control values for animal pairs #9, 10, 16, 13, 11, and 12 is significant ($P < 0.01$). Dose of BSA = 0.6 mg; $B_{12}Co^{60}$ = 0.05 μg. Radioactivity measured in well scintillation detector after sacrificing guinea pig and amputating foot.

ing in inj. foot.

FIG. 3. As for Fig. 2.

FIG. 4. Nine immune and 9 control guinea pigs inj. with $BSA\,I^{131}$ + HGG in right hind foot and $HGG\,I^{131}$ + BSA in left. Each line shows the mean of *in vivo* determination of radioactivity remaining in inj. foot. Dose of BSA and HGG = 0.6 mg of each in each inj. foot.

TABLE III. Comparison of Amount of Antigen and Non-antigen Protein Remaining at Injection Site in Immune and Control Animals.

Animal pair No.	Right hind foot (inj. with BSA I^{131} + HGG)		Left hind foot (inj. with HGG I^{131} + BSA)	
	Immune	Control	Immune	Control
19	36	12	25	14
20	25	14	24	15
21	14	16	10	17
22	20	19	14	21
23	28	14	15	12
24	17	14	20	13
25	22	8	16	12
26	31	9	12	12
27	20	12	13	18
Mean	24	13	17	15

In vitro determination of radioactivity remaining (% of inj. dose) in foot of 9 immune and 9 control guinea pigs sacrificed at 7 hr. T test indicates a significant difference ($P = <0.01$) between radioactivity remaining in right hind foot of immune and control animals. Dose of BSA = 0.6 mg; HGG = 0.6 mg in each rear foot.

injection of a mixture of antigen + $B_{12}Co^{60}$. Fig. 2 and 3 show the *in vivo* radioactivity depicting rate of removal of Vit. $B_{12}Co^{60}$ from injection site in 2 immune animals and 2 controls. Table II shows the remaining radioactivity of Vit. $B_{12}Co^{60}$ counted *in vitro* at time of sacrifice.

b) The effect of the Arthus phenomenon seen on the water-soluble small molecular weight compound was not noticed on the $HGG^{I^{131}}$ during a similar period of time. From Table III and Fig. 4 it can be seen that there is a very minimal difference in rate of elimination of $HGG^{I^{131}}$ in immune and control animals when it is injected along with BSA.

III. Effect of HGG on disappearance of $BSA^{I^{131}}$ from injection site in non-immune guinea pigs:

From Table I one might deduce that addition of HGG to the $BSA^{I^{131}}$ solution delayed the absorption of the latter from the injection site in non-immune animals. Control guinea pigs 18 and 14, injected only with $BSA^{I^{131}}$, showed 2% and 4% of the injected $BSA^{I^{131}}$ remaining at 7 hours, whereas control guinea pigs No. 19 through 27, injected with a mixture of $BSA^{I^{131}}$ + HGG showed a much higher percentage of radioactivity remaining in the foot. However, when this was specifically tested in 4 additional non-immune guinea pigs, no effect of HGG in removal of BSA was discernible (Table IV).

Discussion. Lewis(10), Field and Drinker (11), and Barnes and Trueta(12) have demonstrated that substances with small molecular weight are absorbed more rapidly from the subcutaneous tissue than high molecular weight substances. We have noted the same results: When control guinea pigs were injected with a mixture of $B_{12}Co^{60}$ and $BSA^{I^{131}}$, Vit. B_{12} left the injection site more rapidly than BSA (Fig. 2, 3).

When the same mixture was injected into immune animals there was a delay in removal of $B_{12}Co^{60}$ compared to that in the control (Table III, Fig. 2, 3), and removal of the antigen ($BSA^{I^{131}}$) was much delayed compared to controls. It is apparent that the delay in elimination of $B_{12}Co^{60}$ from the injection site in the immune animals was due to the non-specific mechanical effect of the immune inflammatory process. On teleological considerations it would appear advantageous to have small molecular weight non-antigen materials (*e.g.*, toxins) held at the site of an Arthus reaction. It is likely that the delay in elimination of the antigen was primarily due to the antibodies but was contributed to by the non-specific effect of inflammation. Rich and McKee(13), depriving immune animals of their leukocytes, have pointed out that these animals were able to resist septicemia, at least temporarily, in the absence of any local inflammatory process, when pneumococci were injected intradermally.

Opie(14), has suggested that the specific inflammation at injection site is the result of

TABLE IV. Amount of BSA I^{131} Remaining at 7 Hr at Injection Site of Non-immune Guinea Pigs.

Animal No.	Right hind foot inj. with BSA I^{131}	Left hind foot inj. with BSA I^{131} + HGG
28	9	8
29	8	7
30	6	5
31	8	7

Radioactivity remaining (% of inj. dose) in foot of 4 non-immune guinea pigs inj. with 0.5 mg of BSA I^{131} in right foot, and 0.5 mg of a mixture of BSA I^{131} + HGG, in equal amounts, in the left.

the meeting of antigen and antibody in the tissues. The appearance of this inflammatory process probably further delays antigen elimination.

In our experiments, when serum was tested for radioactivity due to subcutaneously injected antigen, it was found that radioactivity in the serum of immune animals was always less than that of the control. This finding might have been anticipated from reports (7,8) demonstrating an accelerated rate of disappearance of intravenously injected antigen in immune animals. Data reported above show that less subcutaneous antigen reaches the blood stream in immune animals during the first 24 hours after injection.

Summary. An Arthus phenomenon delays removal of the antigen from the area. This is believed to be primarily due to the presence of antibodies. However, a non-specific effect due to inflammation must also be contributory to this slower removal of antigen because removal of a non-antigen from the Arthus reaction area is decreased.

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Prostatic Fraction of Acid Phosphatase in Human Serum.* (26842)

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There is evidence for non-identity of acid phosphatase from different tissues of the same species(1). Heterogeneity of this enzyme has been observed also within the same tissue where it may exist in several distinct forms(2,3). In serum, the level of enzyme activity represents the sum of several components, the characterization of which may be of diagnostic interest. Thus, the Fishman-Lerner method to measure that part of the activity in human serum which derives from the prostate gland has proved very useful in diagnosis of prostatic cancer. In fact, many clinical investigations demonstrate that most of the acid phosphatase of serum, which can be inhibited by L-tartrate, originates from the prostate gland(4,5,6). In this paper it will be shown that the tartrate sensitive fraction of acid phosphatase can be separated from the non-sensitive one by chromatography of serum proteins on anion exchange cellulose columns, and that the tartrate sensitive fraction has chromatographic properties similar to the enzyme extracted from prostate tissue.

Materials and methods. Three samples of normal serum from young healthy males, 4 samples of serum from patients with benign

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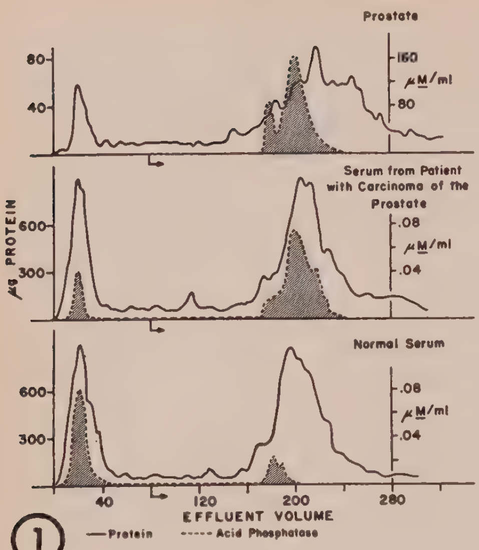

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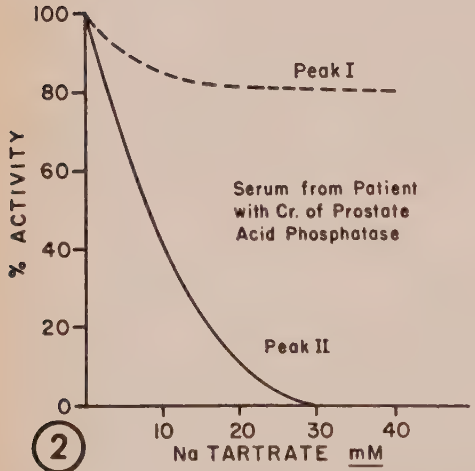

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FIG. 1. Typical chromatograms on DEAE cellulose columns of normal serum, serum from patients with prostatic cancer and of soluble proteins extracted from prostatic tissue. Solid line is protein and broken line is acid phosphatase activity in µmoles/ml/hr of p-nitrophenol liberated from substrate at 38°C.

FIG. 2. Effect of L-tartrate on activity of the 2 peaks of serum acid phosphatase.

hyperplasia of the prostate and 4 samples of serum from patients with prostatic carcinoma were chromatographed. In 2 of the carcinoma cases serum was taken immediately before and one month following surgical operations. Three samples of prostatic tissue, one with prostatic carcinoma and 2 with benign hyperplasia, were also chromatographed.

Prostatic tissue was homogenized in 0.005 M tris-phosphate buffer pH 8.0 with a "Virtis" homogenizer and was then centrifuged at 13,000 rpm for 30 minutes. The clear supernatant from this extract and the samples of serum were dialyzed for 24 hours against the same buffer and stored frozen until used. Column chromatography was carried out on DEAE cellulose as previously described(7). A parabolic gradient elution to a final concentration of 1 M NaCl was used. Proteins were assayed in the fractions by the Lowry method(8). Acid phosphatase was measured in the fractions using p-nitro-phenylphosphate as the substrate(9). Protein recovery was about 80% and recovery for the enzyme activity after chromatography ranged between 70% and 80%.

Results. Fig. 1 shows typcial chromatograms of prostate tissue extracts, normal serum and serum from patients with prostatic carcinoma. Part of the protein was not bound to the column and was eluted directly by the starting buffer. The rest of the protein was then eluted by the gradient and separated into a number of peaks. The acid phosphatase activity in prostate tissue chromatograms was localized in 2 peaks between 0.07 and 0.16 M NaCl. In the chromatograms of normal serum the enzyme was fractionated into 2 main components. One corresponding to about 70%-80% of total activity was found with the protein fraction not bound to the DEAE, and a second one appeared after the gradient elution in a zone between 0.07 and 0.10 M NaCl. Two major components were also found in the serum from patients with prostatic carcinoma. In all these cases, however, the second fraction was spread out in a larger zone and represented from 60% to 80% of total activity. Localization of this more tightly bound fraction of serum acid phosphatase corresponded to the localization of the enzyme in the prostatic extract chromatogram (Fig. 1). As has been reported, prostatic acid phosphatase is completely inhibited by 20 mM sodium tartrate. Fig. 2 shows that the 2 chromatographically separated fractions of serum acid phosphatase have different sensitivities to tartrate. Where-

TABLE I. Distribution of Acid Phosphatase Fractions in Serum.

	% of total activity	
	1st peak	2nd peak
Normal serum	83	17
	72	28
	77	23
Serum from patients with benign hyperplasia of prostate	72	28
	58	42
	70	30
Serum from patients with carcinoma of prostate	32	68
	27	73
	41	59

as the first fraction was only slightly inhibited, the second was completely inhibited at concentration of 30 mM sodium tartrate.

The pattern of distribution of acid phosphatase in serum from patients with benign hyperplasia of the prostate was similar to that of normal serum. Two major fractions were found and most of the enzyme activity was associated with the first unbound protein peak, except for one case in which activity was evenly distributed between the 2 peaks (Table I). After rechromatography under the same conditions, each of the 2 peaks of enzyme activity appeared at the same position as during the first fractionation. In 2 cases of patients with prostatic carcinoma, samples of serum were taken one month after surgical removal of the prostate and subsequent estrogen treatment. In both cases the chromatographic pattern of acid phosphatase returned to normal. That is, the relative amount of activity in the second peak decreased to the normal proportion. The similar chromatographic behavior of the prostatic enzyme and of the second serum component, as well as their equal sensitivity to tartrate inhibition, indicate the probable prostatic origin of this fraction of the enzyme present in the serum. The marked increase of the second peak of serum acid phosphatase in patients with prostatic cancer, followed by a sharp fall soon after prostatectomy, gives further evidence that this peak corresponds to the prostatic fraction of serum phosphatase as determined by the Fishman-Lerner method.

Summary. Serum acid phosphatase represents a mixture of different molecular forms of the enzyme which can be separated and characterized. At least 2 major components can be separated by column chromatography with high recovery of the enzyme activity. The prostatic fraction of acid phosphatase appears, on the basis of its chromatographic behavior and its sensitivity to tartrate inhibition, to be localized in the second chromatographic peak.

Grateful acknowledgement is made of the assistance of Drs. Justin J. Cardonnier and Morris Abrams for collecting tissue and blood used in this experiment.

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Clinical and Serologic Responses in Volunteers Given Vacuolating Virus (SV_{40}) by Respiratory Route. (26843)

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During studies on experimental infection in adult volunteers with respiratory syncitial (RS) virus(1,2) grown in cultures of rhesus monkey kidney cells it was found that the RS virus pool prepared for use in this work was contaminated with vacuolating virus (SV_{40})(3). This paper describes inoculation by the respiratory route of volunteers with the dually infected inoculum and also inoculation with the dually infected material in which RS component had been neutralized by addition of RS antiserum, reports recovery of SV_{40} from throat swab specimens obtained from inoculated volunteers, and presents serologic data indicating that SV_{40}, when administered by the respiratory route, evokes subclinical infection in man.

Materials and methods. Volunteers. Thirty-five test and 11 control subjects employed were male volunteers between the ages of 21 and 35 years. All were given physical examinations including chest roentgenograms, blood and urine tests before and periodically after exposure to test preparations. For several days before and for 2 weeks after inoculation, all volunteers were isolated, usually 3 to a room in the Clinical Center at the Nat. Inst. of Health, under careful medical surveillance; thereafter, they were available for follow-up studies on an out patient basis.

Virus and virus administration. SV_{40} virus employed in the laboratory aspects of the current study was isolated as a contaminant from a pool of RS virus which had been grown in bottle cultures of rhesus monkey kidney cells. Preparation of the RS virus pool is described in detail elsewhere(4). Briefly, 6 to 11 days after inoculation of RS virus, at a time when specific RS cytopathogenic changes were noted in about 50% of the cells in an inoculated culture, the infected cultures were harvested, and the supernatant fluid obtained following low speed centrifugation was filtered through a 830 mμ gradacol membrane and the filtrate stored in convenient amounts at –60°C until used. This material contained in addition to 320 $TCID_{50}$ of RS virus, 5000 $TCID_{50}$ of SV_{40} virus per ml as measured in grivet kidney cell cultures. It may be noted that RS virus unlike SV_{40} does not readily produce cytopathogenic changes in grivet kidney cell cultures. In one volunteer experiment, the RS component of a sample of the dually infected inoculum was neutralized by addition of undiluted guinea pig serum containing RS antibody and in another sample of the infected material the SV_{40} was neutralized by use of guinea pig serum containing SV_{40} antibody. Infectivity titrations performed before and after addition of the antisera showed that neutralization of the homologous agent was accomplished with no appreciable loss of heterologous virus.

Infected and control (Hanks' BSS) inocula were administered to volunteers by the respiratory route. Approximately 1 ml of the original RS-SV_{40} inoculum was nebulized into the nose and mouth, and with the volunteer in a prone position, an additional 0.5 ml was dropped into each nostril. The nose was massaged between thumb and forefinger by the volunteer following inoculation. After this manipulation excess liquid was discharged. In the experiment employing the virus pool to which was added either monotypic SV_{40} or monotypic RS guinea pig antiserum, the volume of the test material administered was increased to 3.0 ml (2 ml of virus mixture + 1.0 ml of serum) to permit the volunteer to receive the full dosage of 10,000 $TCID_{50}$ of SV_{40} virus, or the full dosage of 640 $TCID_{50}$ of RS virus. In presence of the specific SV_{40} antiserum complete suppression of SV_{40} cytopathogenic changes was demonstrated in tube cultures of grivet kidney cells; similarly, in presence of the RS antiserum, RS virus was completely neutralized in cultures of rhesus kidney and Hep-2 cells.

Safety tests. Aerobic and anaerobic cultures of a sample of the dually infected pool on blood agar and thioglycolate medium failed to show the presence of bacterial contaminants. Tests to detect extraneous viruses in the pool material gave negative results in rabbit kidney cultures; no significant cytopathogenic changes occurred during 3 weeks following inoculation. Rabbits, mice and guinea pigs inoculated by multiple routes with the test material remained free of signs of illness during a 3-week observation period. Inoculation of the test pool into cultures of grivet kidney cells demonstrated the presence of SV_{40} virus. Detailed account of safety tests is given elsewhere(4).

Virus recovery. SV_{40} virus isolation attempts were performed in duplicate tube cultures of grivet kidney cells. Throat and rectal swab specimens were collected just prior to and daily (throat swabs) or every other day (rectal swabs) for 12 days after administration of test and control preparations. The swabs were immediately immersed in 3 ml of Hanks' salt solution containing 0.5% gelatin. After storage for a few days to several weeks at –60°C in plastic capped glass vials, 0.2 ml amounts of each specimen were inoculated into grivet kidney cell cultures. If no specific cytopathogenic effect was observed during 21 days of incubation at 36°C the cells and fluids were passed to new tubes: when such subcultures failed to show changes characteristic of SV_{40} infection the isolation attempt was considered negative. Recovered virus was identified in neutralization tests with 2 antisera, one prepared by us in guinea pigs inoculated with the strain of SV_{40} virus recovered from the original RS virus pool and the other in Dr. Hilleman's laboratory against the prototype strain of SV_{40} virus.

Virus identity tests. Neutralization tests using monotypic guinea pig sera were carried out in Hep-2 and rhesus kidney or grivet kidney cell cultures as described(4); these served to identify the presence of RS and SV_{40} viruses in the dually infected inoculum and showed the absence of other detectable viruses.

Neutralization tests. Serial 2-fold dilutions of serum (inactivated 56°C for 30 minutes) in 0.5 ml amounts were mixed with approximately 250 $TCID_{50}$ of SV_{40} virus contained in 0.5 ml of infected tissue culture material. The mixtures were incubated in a water bath at 37°C for 30 minutes after which 0.4 ml of each mixture which contained about 100 $TCID_{50}$ of virus was added to each of 2 tubes containing grivet kidney cells. The cultures were examined on the 10th day for presence or absence of specific vacuolating effect characteristic of SV_{40} infection. Serum titers are expressed as the highest initial dilution of serum, before addition to equal volume of virus and inoculation into cell culture tubes containing 1.0 ml nutrient-fluid, which completely inhibited specific cellular degeneration.

Results. Clinical evidence of SV_{40} infection. All 8 volunteers inoculated by the respiratory route with a preparation containing 10,000 $TCID_{50}$ of SV_{40} virus in which the RS virus component had been neutralized by RS antiserum remained free of signs and symptoms of illness in the month following inoculation. Two of 8 subjects inoculated by the same route with the RS preparation in which the SV_{40} component had been neutralized by SV_{40} antiserum developed respiratory disease (4). Sixteen of 27 individuals inoculated with a mixture of 640 $TCID_{50}$ of RS virus and 10,000 units of SV_{40} experienced respiratory disease described in detail elsewhere(4), none of the 27 test subjects, however, developed signs or symptoms which differed from those observed in volunteers who received the RS virus mixture in which the SV_{40} component had been neutralized.

Serologic. Neutralizing antibody responses elicited in 35 test subjects by inhalation of 10,000 $TCID_{50}$ of SV_{40} virus are given in Tables I and II. All preinoculation sera obtained from volunteers listed in Tables I and II were devoid of SV_{40} neutralizing antibody at a 1:5 dilution. All 8 recipients of the dually infected material in which the RS constituent was neutralized by addition of RS antibody, developed SV_{40} neutralizing antibody in titers ranging from 1:5 to $>$1:20 (Table I). Fourteen of the 27 volunteers given inoculum containing both viruses in the active, unneutralized state developed neutralizing antibody against the SV_{40} component in titers comparable to those seen in the volunteers receiving material in which the RS constituent had been neutralized with specific RS antiserum (Table II). None of the 8 subjects given the dual material mixed with SV_{40} antiserum developed SV_{40} antibody during the month following inoculation. Six of the persons listed in Tables I and II who developed SV_{40} antibody following inoculation supplied additional sera 6-9 months later. Four of these still possessed antibody of the same or lower titers; 2, whose original titers had been low, no longer possessed detectable antibody.

Virus isolation. From throat swabs of 2 of the test subjects, SV_{40} virus was recovered on the 7th day, and from another, on the 11th day (Table I). None of the throat or rectal swab specimens taken at other times yielded SV_{40} virus. Each of the 3 isolates was shown to be identical with SV_{40} in neutralization tests with specific antiserum provided by Dr. Hilleman and with antiserum prepared in our laboratory in guinea pigs against the SV_{40} strain recovered from rhesus monkey kidney cell preparation infected with RS virus. All 3 isolates were recovered in cell cultures inoculated with the original clinical specimens. Blind passage attempts failed to yield additional isolates.

TABLE I. Virus Isolation and Serologic Findings in Volunteers Inoculated with Dually Infected Inoculum to Which Was Added Guinea Pig Serum Containing Antibody against One or the Other of the Infected Viruses.

		SV_{40} virus isolation	SV_{40} neutralizing antibody titer	
Inoculum*	Volunteer	Throat swab	3 wk	5 wk
SV_{40} and RS viruses + RS antiserum	1	Pos. day 7	>20	ND
	2	" " 7	>20	"
	3	" " 11	20	"
	4	Neg.	10	"
	5	"	10	"
	6	"	10	"
	7	"	10	10
	8	"	5	5
SV_{40} and RS viruses + SV_{40} antiserum	9	Neg.	0	ND
	10	"	0	"
	11	"	0	"
	12	"	0	"
	13	"	0	"
	14	"	0	"
	15	"	0	"
	16	"	0	"

0 = No neutralization at 1:5 serum dilution, the lowest dilution tested.

* In each instance the virus mixture contained 10,000 $TCID_{50}$ of SV_{40} virus and 640 $TCID_{50}$ of RS virus.

TABLE II. Virus Isolation and Serologic Findings in Volunteers Inoculated with SV_{40} and RS Viruses and Control Preparation.

		SV_{40} neutralizing antibody titer	
Inoculum*	Volunteer	3 wk	34-38 wk
RS + SV_{40} viruses	17	80	20
	18	80	5
	19	>20	ND
	20	10	"
	21	10	"
	22	5	0
	23	5	0
	24	5	ND
	25	0	"
	26	0	"
	27	0	"
	28	0	"
Hanks' BSS	29	0	"
	30	0	"
	31	0	"
RS + SV_{40} viruses	32	>20	ND
	33	20	"
	34	20	"
	35	10	"
	36	5	"
	37	5	"
	38	0	"
	39	0	"
	40	0	"
	41	0	"
	42	0	"
	43	0	"
	44	0	"
	45	0	"
	46	0	"

0 = No neutralization at 1:5 serum dilution, the lowest dilution tested.

* In each instance the virus mixture contained 10,000 $TCID_{50}$ of SV_{40} virus and 640 $TCID_{50}$ of RS virus.

Discussion. Our interest in SV_{40} is centered around its potentialities as an infectious agent for man. Sabin has reported evidence which leads him to conclude that SV_{40} when administered orally fails to infect man(5).*

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* Our studies on materials made available to us by Dr. Sabin support his idea. These materials consisted of a sample of type 1, live, attenuated, oral, polio vaccine, strain LSC, which had been administered to 12 children whose paired sera were also submitted to us for examination in SV_{40} neutralizing antibody tests. The vaccine contained at least 500

The present experience provides data suggesting that SV_{40} when given by the respiratory route induces subclinical infection in man, manifested by presence of virus in the throats of a few of the subjects 7 to 11 days after exposure, and by development of specific neutralizing antibody in about two-thirds of the volunteers in the weeks following inoculation. The generally low levels of serum antibody detected in the volunteers after exposure to SV_{40} and failure to recover virus from most of the volunteers in the present work suggest that SV_{40} induces only a low grade infection in adults when introduced by the respiratory route.

Summary. SV_{40} virus present as a contaminant in a pool of respiratory syncitial virus prepared in cultures of rhesus monkey kidney cells was inoculated by the respiratory route into 35 adult volunteers: 8 of these received SV_{40} virus preparation in which the RS virus component was neutralized by addition of RS antiserum. None of the test subjects developed signs or symptoms attributable to inhalation of SV_{40}. SV_{40} virus was recovered from throat swab specimens of 3 of the volunteers 7 or 11 days after exposure. SV_{40} neutralizing antibody in titers ranging from 1:5 to 1:80 appeared in the blood of 22 of the 35 test subjects.

$TCID_{50}$ of SV_{40} per ml. None of the 12 pairs of serum, however, contained SV_{40} neutralizing antibody when examined at a 1:5 dilution against 100 $TCID_{50}$ of our strain of SV_{40} virus.

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Effect of Acid Mucopolysaccharides on Hair Growth in the Rabbit.* (26844)

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Histologic studies on the cyclic hair growth in animals have shown a close correlation between activity of the hair follicles and metachromatically staining material in and around the hair bulb(1). The same areas are heavily labeled on injection of $S^{35}O_4$ in the rat(2), though not in the mouse(3). The nature of the presumed sulfated mucopolysaccharide (MPS) has never been determined. The starting point of the present studies was the observation that most, if not all, children with Hurler's syndrome had extremely thick hair and appeared to have a faster rate of hair growth than children of comparable age. Since in Hurler's syndrome there is a characteristic storage and urinary excretion of 2 sulfated polysaccharides, chondroitin sulfate B and heparitin sulfate(4), the possible effect of intradermal injection of these and other polysaccharides on hair was studied in pigmented adult rabbits.

Experimental. The rabbits were kept in individual cages and fed a standard laboratory diet. The hair of the back was clipped. The rabbits were used only if the underlying skin was uniformly unpigmented, to insure as much as possible that all areas of the skin were approximately at the same stage of the hair cycle(5). Several areas of about 2 × 2 cm were marked off in each rabbit. 0.5 ml of different solutions of polysaccharide in

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† Fellow, Arthritis and Rheumatism Foundation.

TABLE I. Total of 34 Injection Sites on 8 Different Rabbits, Each Site Injected Daily with 0.5 ml of Substance Indicated at Concentration of 1.0 mg/ml.

	Substance								
	Hep.S.	ChS-B	H.A.	ChS-C	ChS-A	NaCl	Air	Calcium alginate	Ovalbumin
No. of inj. sites	7	8	6	2	2	4	1	2	2
Avg rating	2.9	1.6	1.0	1.0	.5	0	0	0	0

Rating: 0 = no growth; 1 = min growth; 2 = mod growth; 3 = max growth.

TABLE II. Each Rabbit Received One ml of the Indicated Suspension or Solution Intracutaneously. Hair growth evaluated 3 to 4 weeks after injection. Substances 1 to 6 are suspensions made up by mixing 0.5 ml of polysaccharide solution at 20 mg/ml with 0.5 ml of protamine sulfate solution at 20 mg/ml.

	Substance inj.									
Rabbit	1. Hep.S.–prot. sulf.	2. ChS-B–prot. sulf.	3. 1:1 mixture of 1 & 2	4. H.A.–prot. sulf.	5. ChS-C–prot. sulf.	6. Ca alginate–prot. sulf.	7. ChS-B, 20 mg/ml	8. Hep.S., 20 mg/ml	9. Protamine sulfate, 20 mg/ml	10. 1:1 mixture of 7 & 8
1	2	0		1	0	0				
2	2	0		1	1	1				
3	2		1				0	0	0	0
4	0		2				0	0	0	0
5	2			1	0					
6	2			1	1	0				

Rating: 0 = no growth; 1 = intermediate growth; 2 = maximum growth.

a concentration of 1 mg/ml were injected intracutaneously into the marked areas. A total of 34 different injection sites was used in 8 rabbits. In the first experiments, injections were made daily for periods of 3 to 7 weeks or until there was obvious regrowth of hair in at least one injection site. The MPSs used[‡] were purified in this laboratory by standard procedures(6). The results of this experiment are shown in Table I. Rating of the effect was based on number, length, thickness and intensity of pigmentation of the hair in injected sites. 0 indicates no visible effect as compared to a non-injected site. Maximum effect was scored as 3. All the injected MPSs exhibited some stimulation of hair growth. However, Hep. S. was more potent than any other polysaccharide, followed by ChS-B. Physiological saline, air, Ca alginate (1 mg/ml) or ovalbumin (1 mg/ml) had no effect. In 6 out of 7 sites, the effect of Hep. S. was maximal. The effect is illustrated in a composite photograph of 4 areas in one rabbit 4 weeks after start of injections. This shows the types and intensities of the reaction (Fig. 1). Scores of the sites were recorded as A = 1, B = 2, C = 3, D = 0.

To provide a depot of the MPSs, suspensions of mucopolysaccharide and protamine sulfate were made up as described in Table II, and 1 ml was given intracutaneously only once. In this experiment Hep. S. again was most effective. Injection of twice the concentration of polysaccharides without protamine sulfate or protamine sulfate alone had no effect.

The effect of Hep. S. in one rabbit is shown in Fig. 2. Attempts to reproduce these effects in albino rabbits gave inconclusive results. Plucking instead of clipping hair prior to injection caused intense inflammatory reactions followed by rapid regrowth of the hair within one week.

Biopsy specimens were obtained from the injection areas of the skin of 6 of the 14 rabbits, at least 3 days following last injection. In the case of the MPS-protamine complex,

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[‡] The following abbreviations are used: Chondroitin Sulfate = ChS; Hyaluronic Acid = H. A.; Heparitin Sulfate = Hep. S.

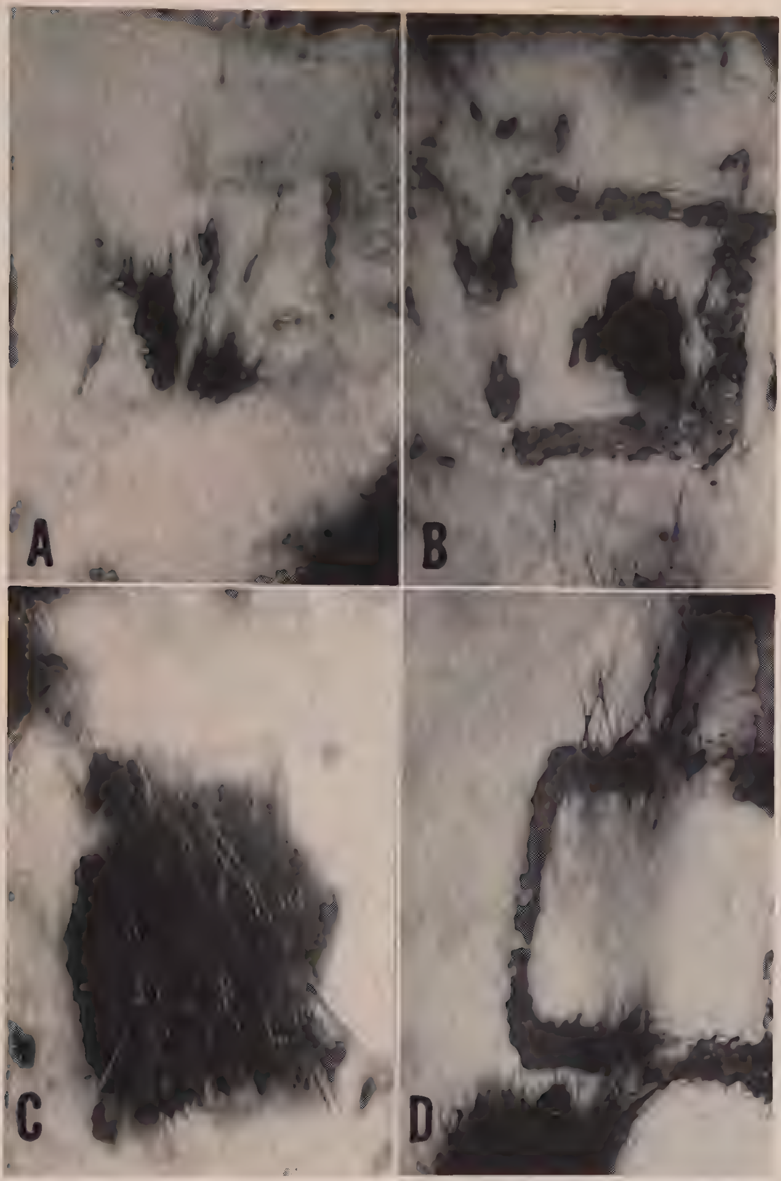

FIG. 1. Four inj. sites on back of shaved rabbit 4 wk after daily inj. of following substances in dose of 0.5 ml/day. A = ChS-A, B = H.A., C = Hep. S., D = Saline.

the time interval was 4 weeks after the single injection. Each area was divided into 2 portions, one-half being fixed in 10% formalin or Bouin's solution, the other in 96% alcohol. A typical example of the tissue reaction is pictured in Fig. 3. This picture was chosen since it shows an uninjected area adjacent to an area injected in this case with ChS-B daily for 4 weeks.

In control areas and in areas receiving H.A., no active hair growth or only a few actively growing hairs were found in the sections. In areas injected with Hep. S. and ChS-B, or in some with ChS-A, a most pronounced growth was seen histologically with the hairs thicker than normal. The papillae of some hairs were unusually large and contained numerous large histiocytes. In addition, some inflammation was seen in the corium and in the epidermis of all specimens.

The growth of single hairs in the control areas was apparently caused by plucking of these hairs during clipping.

Discussion. The pattern of hair growth undoubtedly is a complex phenomenon. The role of pigmentation and inflammation in the hair growth of the colored rabbit has been well described(5). According to the report of these authors, inflammation accompanied by crusting and scaling of the skin always is associated with effective chemical "irritation" and stimulation of the resting hair follicles. Hyperemia and erythema unassociated with crusting and scaling, as produced by mild irritants, did not initiate a hair growth cycle in the rabbit. In our experiments, no crusting or scaling of the skin during or after the injections was observed.

The correlation of active hair growth with presence in the dermal papilla of metachromatically staining material, presumably a sulfated MPS resistant to testicular hyaluronidase, has been well documented(7). It appears from the data reported here that sulfated MPSs and especially Hep. S., on intradermal injection, are able to initiate growth activity in quiescent hair follicles of the pigmented rabbit. We assume that metachromatic cells in and around the papilla normally produce similar substances and that inflammation acts similarly *via* cells producing metachromatic substances. Conversely prolonged treatment with heparin or especially heparinoids has been reported to lead to partial or total alopecia in a very high percentage of cases (in 99% of cases treated with a dextran sulfate(8)). These sulfated polysaccharides can be assumed to inhibit the reactions of the normal polysaccharide. The nature of the reaction or reactions involved in activation of the hair follicle is unknown. It certainly is not the furnishing of S-containing precursors of keratin suggested by Sylvén(9).

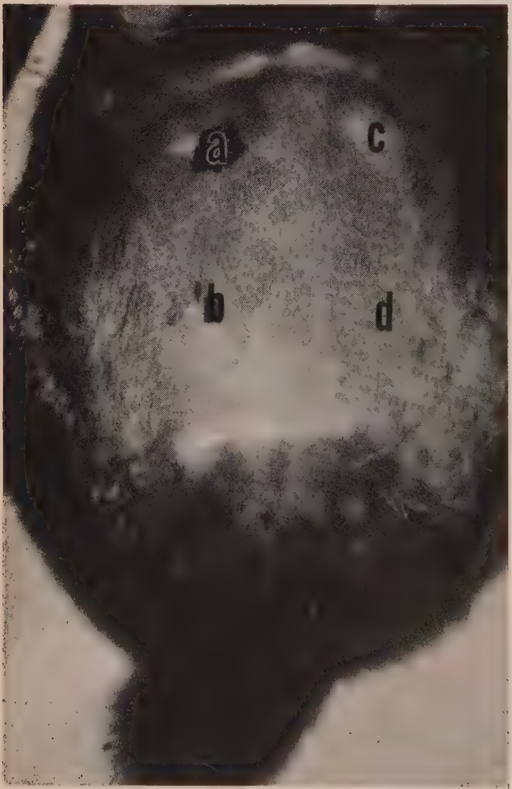

FIG. 2. Effect of 1 inj. of MPS-Protamine complex (10 mg of each) after 4 wk on the back of a shaved rabbit: a = Hep. S., b = ChS-A, c = saline, d = no inj.

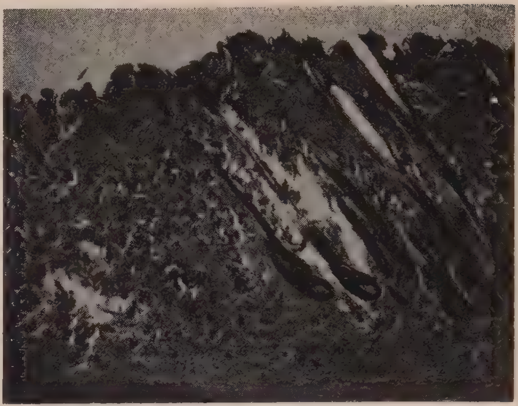

FIG. 3. Hematoxylin and Eosin section of rabbit skin showing uninj. area on left, and area inj. with ChS-B on right, containing many large, active hair follicles.

Summary. Repeated intradermal injection of MPS initiated a hair growth cycle in the pigmented rabbit. Hep. S. was the most active in the reaction, followed by ChS-B. A single injection of MPS applied as the insoluble protamine complexes also stimulated hair growth, again with Hep. S. the most active. These experiments suggest an essential role of a sulfated MPS in the normal hair growth cycle, as suggested previously from

the correlation of metachromasia and hair growth.

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Disappearance of Human Diphtheria Antitoxin from Human Passive Transfer Skin Sites.* (26845)

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Kuhns(1) described a method for measuring the disappearance of human diphtheria antitoxin from passive transfer skin sites. The tests are carried out in Schick positive persons, and they depend upon intradermal antitoxin totally or partially to neutralize Schick toxin which is introduced later. Subsequent partial or total inhibition of toxic reactions indicate the degree of disappearance of antitoxin from skin sites. Further experiences with this method are described here. Precipitating, non-precipitating and skin sensitizing human antitoxic sera are compared as to their disappearance from passive transfer sites.

Materials and methods. Materials used in the Schick tests, evaluation of immediate reactions to Schick toxoid, and methods of hyperimmunization of Schick negative subjects have been described(2). Preparation and properties of the diphtheria toxin and toxoid used and the technics employed in carrying out the intracutaneous neutralization test in rabbits are described elsewhere(2,3,4). Human immune sera contained high titers of antitoxin as measured by rabbit skin tests. Precipitating and non-precipitating sera, and skin sensitizing and non-sensitizing sera have been used for purposes of comparison. Specimens from 6 individuals were utilized, designated as follows: Sp (non-precipitating – 15 Units/cc), She (precipitating – 80 Units/cc), Rie (precipitating – 50 Units/cc), Ni (non-precipitating – 100 Units/cc), Bo (precipitating skin sensitizing – 12 Units/cc), and Hu (non-precipitating skin sensitizing – 20 Units/cc). All serum samples to be tested were removed from the frozen state at 0°F and rapidly thawed to room temperature just prior to use. Dilutions of antitoxin were made in borate-NaCl buffer pH 7.4 containing 200 μg gelatin/cc to prevent surface denaturation. Methods of quantitatively measuring precipitation in a fluid and semi-solid medium have been described(5). Wheal reactivity was measured by the method of passive transfer (6). Toxoid and toxin preparations from the same source were employed for all testing purposes.

Neutralization of Schick Toxin by Intradermal Antitoxin: This test is based upon the ability of intradermal antitoxin to neutralize the toxic action of the equivalent amount of Schick toxin in Schick positive subjects. Recipients were used for no more

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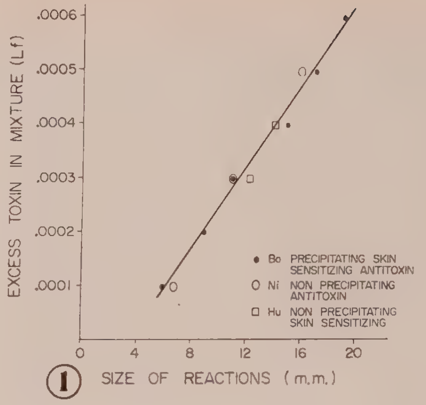
† The author wishes to express his gratitude to Dr. James McComb, Mass. Antitoxin Laboratory, Jamaica Plains, Mass., for providing purified diphtheria toxoid (KP 28 D5) and Shick testing outfits used in these experiments.

than 2 series of tests spaced about one week apart. Circulating antitoxin titers were carried out on blood specimens obtained at time of testing. Recipients who demonstrated a changing circulating antitoxin titer were not employed for additional tests. When successive series of passive transfer tests were carried out in one person, sites of earlier testing were avoided. Excellent agreement in results was obtained in testing in duplicate skins when both recipients were Schick positive.

Skin disappearance of antitoxin was measured as follows: Control experiments were run in which separate mixtures containing both Schick toxin (0.0006 Lf) and different amounts of antitoxin (0.0001 to 0.0012 Unit) were placed at separate intradermal sites following incubation at 37°C for one hour. If toxic reactions occurred, measurements were made (diameter in mm) 4 days later. Each measurement provided an indication of the free toxin which remained in such mixtures, permitting direct correlation of this information with size and intensity of the toxic reaction. The data in turn were applied to other experiments where first antitoxin and then toxin were injected at variously spaced intervals at the same sites. A toxic reaction measured as described above served as an indication of toxin in excess of the amount which was bound to intradermal antitoxin. The amount of antitoxin bound to toxin in the skin was then estimated assuming 1 Lf = 1 Unit of antitoxin. On the basis of difference it was also possible to estimate the amount of antitoxin which disappeared between sensitization and antigen challenge at the same site. In the present experiments, the amount of antitoxin used to sensitize a skin ranged from 0.0003 Unit to 1.2 Unit contained in 0.1 cc. Challenge with 0.1 cc (0.0006 Lf) of Schick toxin was at intervals ranging from 6 hours to 7 days.

For each antitoxic serum or serum dilution which was injected alone prior to toxin challenge, a control containing the same amount of antitoxin in a mixture with toxin was injected simultaneously in the same recipient.

Results. A straight line relationship was observed between size of reactions and amount of excess toxin in mixtures containing Schick toxin (0.0006 Lf) and different amounts of antitoxin ranging from 0 to 0.0005 Unit (Fig. 1). Precipitating, non-precipitating and skin sensitizing antitoxins all exhibited the same relationship when employed at comparable strengths in mixture with toxin. When antitoxin was injected separately followed by toxin later at the same skin sites, reactions undiminished in size and intensity from toxin controls at unsensitized sites were indicative of the disappearance of all antitoxin between sensitization and challenge. Attenuated reactions, depending upon



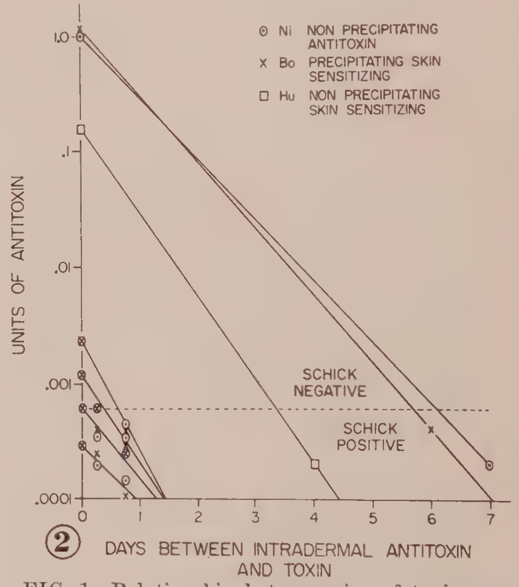


FIG. 1. Relationship between size of toxic reactions and excess toxin in mixtures containing Schick toxin and antitoxins in different amounts.

FIG. 2. The disappearance of human diphtheria antitoxin from human passive transfer sites.

TABLE I. Disappearance of Human Antitoxin Bo in Skin of Schick Positive Recipient Judged According to Ability to Neutralize Schick Toxin.

Units of antitoxin at skin site	Time of challenge with .0006 Lf Schick toxin, hr	Toxic reaction 4 days after challenge, mm intensity	Calculated free toxin (Lf) causing reaction	Calculated antitoxin (units)	
				Remaining at time of challenge	Disappeared between skin test and challenge
.0024	6	0	0	>.0006	<.0018
.0012	”	5 ±	Little or 0	ca. .0006	ca. .0006
.0006	”	9 ++	.0002	.0004	.0002
.0003	”	13 +++	Between .0003 & .0004	Between .0002 & .0003*	.0001
.0024	18	9 +++	.0002	.0004	.0020
.0012	”	11 +++	.0003	.0003	.0009
.0006	”	13 ++++	Between .0003 & .0004	Between .0002 & .0003	Between .0003 & .0004
.0003	”	17 ++++	.0005	.0001	.0002

* A point midway between these figures is selected for purposes of charting.

size and intensity, represented a partial disappearance of antitoxin (Table I).

Rate of disappearance of intradermal antitoxin was determined. In one set of experiments, the amount of intradermal antitoxin very closely approximated the dose of Schick toxin employed later. Accordingly the following amounts of antitoxin were placed at intradermal sites 4 to 5 cm apart on the back in 2 different Schick positive recipients; 0.0003, 0.0006 and 0.0012 Unit. Skin sites were prepared in this fashion in duplicate so that toxin challenge of a single series could be carried out at 6 hours and at 18 hours after sensitization. Toxic reactions at test sites were compared with reactions at control areas (see under Methods) and antitoxin which remained at test sites was estimated as described previously. Results of such an experiment are indicated in Table I. Sites challenged at 6 and 18 hours were found to contain residual antitoxin, but smaller amounts of antitoxin were present upon toxin challenge at 18 hours as compared with challenge at 6 hours. The antitoxin initially utilized, together with antitoxin residuals at 6 and 18 hours were then plotted as a function of time to obtain the rate of disappearance of this antibody. On the basis of the above experiments, it was estimated that 50% of diphtheria antitoxin disappeared from intradermal sites in 10 to 14 hours when 0.0003 and 0.0006 and 0.0012 Units of intradermal antitoxin were used (Fig. 2).

In other experiments, sensitization with intradermal antitoxin was carried out using amounts far in excess of the amount equivalent to the dose of Schick toxin employed later. 0.0024 Unit, 0.15 Unit, 1.0 Unit and 1.2 Units of antitoxin contained in 0.1 cc were placed in replicate spaced intradermal sites in the back of Schick positive subjects. Later challenge with Schick toxin (0.0006 Lf) was made when the residual intradermal antitoxin was believed to be 0.0006 Unit or less (in the case of 0.0024 Unit, times of challenge were 6 and 18 hours). A suitable time interval could usually be extrapolated from experiments in which equivalent antitoxin and toxin were utilized based upon an appropriate multiple of the time required for disappearance of antitoxin under these circumstances (Fig. 2).

Under the conditions of these experiments 50% of the antitoxin disappeared from skin in 10 to 14 hours when 0.15 Unit, 1.0 Unit and 1.2 Units were initially used. A 50% disappearance rate of 7 hours was obtained when 0.0024 Unit of antitoxin was employed.

Non-precipitating antitoxin, precipitating and skin sensitizing antitoxins all behaved similarly in these experiments (Fig. 2, Table I).

Discussion. The disappearance of human diphtheria antitoxin was studied in human passive transfer skin sites using a method which depended upon intradermal antitoxin to neutralize Schick toxin totally or in part.

Schick positive recipients were required for these experiments. Intradermal antitoxin in sufficient amount in these persons produced local areas which were Schick negative. The extent of disappearance of intradermal antitoxin was calculated following reversion to the Schick positive state when comparison of the toxic reaction could be made with reactions produced by standard toxin-antitoxin mixtures. All calculations of antitoxin were made according to the formula 1 Lf toxin = 1 Unit antitoxin. Loss of antitoxin was then estimated by subtracting residual antitoxin (as calculated above) from the antitoxin initially used. Estimates of rate of disappearance were made by plotting initial *vs.* residual antitoxin as a function of time.

It was found that 50% of antitoxin disappeared from skin sites in 10 to 14 hours when the following amounts of intradermal antitoxin were employed; 0.0003 Unit, 0.0006 Unit, 0.0012 Unit, 0.15 Unit, 1.0 Unit and 1.2 Units. A 50% disappearance rate of 7 hours was obtained when 0.0024 Unit was used. On the whole the results employing various amounts of antitoxin were in good agreement, and non-precipitating, precipitating and skin sensitizing antitoxin behaved similarly under conditions of the test.

The tendency to variability in this figure for antitoxin excess may be explained as follows. Under this circumstance the skin was not reactive to Schick toxin during most of the time that intradermal antitoxin was migrating away. Any data on antitoxin disappearance were of necessity derived from gradations in toxic reactions over a short period of time during which time only a small part of the original antitoxin remained. The fraction of antitoxin which remained was not known exactly but was extrapolated from disappearance rates of much smaller *i.e.*, equivalent amount of antitoxin. This ordinarily allowed for a single challenge following sensitization. Curves constructed from these data were thus based on 2 points (Fig. 2). On the other hand, additional points, particularly in the immediate period following sensitization, were possible when intradermal antitoxin in the region of equivalence was employed. Disappearance after 18 hours could not be tested because of difficulties of interpreting small differences between control and sensitization sites.

Other technics and immune systems have been used to study antibody metabolism in skin. Farah, Kern and Eisen(7) studied the disappearance from human skin of purified rabbit anti-DNP antibody and the fragments split from this antibody by papain. Intradermal injections were made using I^{131} labelled antibody or antibody fractions, and counts of radioactivity were used to measure loss of antibody from the skin. The results indicated a half life of approximately 10 hours for intact antibody and antibody fractions. Humphrey, Neuberger and Perkins (8) also utilized radioactive isotopes in experimental animals to calculate the exchange between skin albumin and circulating albumin. Their data suggested that 60% of skin albumin is replaced every day by circulating serum albumin.

The findings for disappearance of antibody in human skin are consistent with our results. The results suggest that, for the systems employed, disappearance of antibody from human skin occurs at a relatively constant rate, and is not apparently related to differences in molecular weight, precipitability, or immediate wheal activity.

The method described here may be useful in further investigations on the nature of reagins. Purified systems must be employed to study further comparisons between the wheal reactive state and other criteria of antibody activity in the skin as described herein, to assess the significance of earlier findings (1) that reagin persists for an unusually long period *in vivo*.

Summary. This paper has described further experiences with a passive transfer technic which depends upon the ability of intradermal antitoxin to neutralize Schick toxin introduced simultaneously or later at the same sites. Partial, total or no inhibition of toxic reactions indicates the amount of antitoxin remaining at the skin sites. The amount of antitoxin which has disappeared may be calculated by difference, assuming that 1 Lf toxin = 1 Unit of antitoxin. On the basis of this test 50% of antitoxin intro-

duced at skin sites was generally found to disappear in 10 to 14 hours. No difference in disappearance from the skin was observed between precipitating antitoxin, non-precipitating antitoxin and skin sensitizing antitoxin.

The author gratefully acknowledges the technical assistance of Mrs. Velma Axelrod.

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Effect of Topical Application of Hydrocortisone on Dermic Lesions Produced in Rabbits by Vaccinia Virus. (26846)

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Cortisone and its derivatives are commonly employed as topical medication in several skin and mucosal diseases. Inflammatory reactions are thereby diminished and symptoms such as rash and pruritus are alleviated in many cases. However, in other cases local application of cortisone involves hazards which it is important to consider. These hazards occur in some viral diseases, among which those produced by herpes simplex virus are good examples. In fact, in man, conjunctival lesions of such an origin grow and deepen as a result of the local action of corticoids, as has been observed in experimental infection of the rabbit(1,2). Trachoma is also aggravated during the acute phase or may be reactivated during later stages when treated locally with those substances(3). Nevertheless, the literature does not mention similar observations in relation to other viruses that produce epidermal lesions.

In this work the local effect of hydrocortisone on dermal lesions produced in the rabbit by vaccinia virus is studied.

Material and methods. The strain employed belonged to the Instituto Nacional de Higiene virus seed whose lots 32 and 38 of vaccinia virus were prepared in calves. In the lymph potency test, determined by Force and Leake's technic(4), confluent lesions were observed at 1:10,000 dilution of lymph and isolated lesions at 1:30,000.

Once the inoculum potency had been determined, 24 healthy albino rabbits were selected at an average weight of 4,500 g. Two areas on the back of each rabbit, measuring 4 cm × 4 cm and situated 5 cm apart, were depilated. One percent hydrocortisone ointment was applied on one of the areas and neutral vaseline on the other. Both applications were performed daily. Six days after the treatment had begun the rabbits were inoculated intracutaneously through scarification with 0.3 ml of a viral suspension diluted 1:30,000 in saline. The inoculum was carefully spread throughout the area. Two hours later, when the fluid had thoroughly soaked the skin, ointments were applied and the treatment was continued for 6 days more.

To detect the appearance of vesicles the rabbits were observed daily until maximal development of lesions. As soon as the vesicles appeared they were measured every 24 hours by applying a glass slide over the affected area in such a way that the outline of the lesions observed through the glass could be traced with India ink. The area of the lesions was determined by setting the slides on millimetric paper. Another group of 12 inoculated rabbits, treated in the described way, was assigned to provide virus for titration from the lesions found in the 2 areas when maximal development was reached. To harvest the virus for titration the areas were first washed with soap and water and the lesions were thoroughly scraped with a scalpel. The material obtained from each area was separately ground in a mortar adding sterile saline until a volume of 10 ml was reached. From this suspension, which was regarded as undiluted, serial dilutions were made with a factor of ½ log, and inoculated at doses of 0.2 ml onto the chorioallantoic membrane of chick embryos (6 per dilution). The eggs were incubated for 72 hr, then kept in the freezer 4 hours more and the chorioallantoic membrane harvested. Titers were estimated by the pock counting method(5). Using 6 tubes per dilution of rhesus monkey kidney cell cultures maintained with 60% Hanks' solution, 20% lactalbumin hydrolysate and 20% normal horse serum, a parallel titration was performed with 0.2 ml of viral suspension. The inoculated tubes were incubated for 30 minutes before adding the culture medium. During the next 10 days the tubes were observed for CPE typical of vaccinia virus infection in this type of cells, as described elsewhere(6,7). Titers were estimated by the Reed and Muench method(8).

Results. On the appearance of lesions, usually 72 hr after inoculation, results were evaluated by comparing the total extent of injured areas in corresponding sites on the 24 rabbits. From the fourth day it became apparent that the size of the injured surface in the areas treated with cortisone was larger than control areas (70% greater). This difference increased in the following days. Measurements taken when the lesions reached maximal development are shown in Table I. There is a significant difference between the extent of affected surface in treated and control areas.

TABLE I. Action of Cortisone on Number and Total Area of Lesions Produced by Vaccinia Virus in 2 Cutaneous Scarifications in 24 Rabbits.*

Rabbits	No. of lesions in area 4 cm × 4 cm		Total injured area, cm²		Diff., cm²
	+ Cort.	− Cort.	+ Cort.	− Cort.	Δ
1	3	4	3.5	2.6	.9
2	4	4	3.8	2.0	1.8
3	5	4	3.6	2.4	1.2
4	3	4	3.4	2.4	1.0
5	4	3	3.4	1.8	1.6
6	4	5	3.8	2.8	1.0
7	3	4	3.2	2.4	.8
8	3	3	3.6	1.5	2.1
9	4	4	3.4	2.2	1.2
10	4	3	3.6	1.6	2.0
11	3	4	3.5	2.0	1.5
12	4	5	3.4	2.5	.9
13	4	4	3.6	1.8	1.8
14	3	4	3.6	1.8	1.8
15	4	4	3.2	2.2	1.0
16	4	3	3.4	1.4	2.0
17	5	4	3.8	2.0	1.8
18	5	4	4.2	2.4	1.8
19	4	3	3.6	1.6	2.0
20	4	4	3.6	1.8	1.8
21	4	4	3.4	1.8	1.6
22	4	5	3.2	1.8	1.4
23	4	4	3.2	2.4	.8
24	5	4	3.8	2.8	1.0
Avg	3.9	3.9	3.5	2.08	1.45
				Total:	34.8

$\bar{\Delta}$ Differs significantly from zero ($p < 0.01$).

* Calculations were made from measurements taken at time the lesions reached maximal development; on the 7th day for control areas and on the 9th for cortisone-treated areas.

It should be pointed out that in control areas the lesions reached maximal development on the seventh day, whereas in areas treated with cortisone the vesicles continued to enlarge until the ninth day. Moreover the involutive phase of cortisone-treated lesions involved 17 days, in comparison to 10 days in the controls. The total process lasted 26 days in areas treated with cortisone and 17 days in control areas, a difference of 9 days. These data can be seen in Fig. 1. After healing, treated and control areas presented different aspects. The site of lesions on the cortisone-treated skin was marked by a small depressed area, while complete restoration to normal appearance of the skin occurred at control sites.

Comparative titrations obtained from virus contents of the lesions found in treated and control areas were performed on the chick embryo chorioallantoic membrane and in

TABLE II. Titers of Material Tested in Embryonated Eggs and in Tissue Cultures.

Type of material	Titration in chorioallantoic membrane	Titration in *M. rhesus* kidney cell cultures
With cortisone	8.2×10^8 infectious particles/ml	$10^{8.3}ID_{50}$ per ml
Without cortisone	8.0×10^8 " " "	$10^{8.1}ID_{50}$ "

rhesus monkey kidney cell cultures. By either method, the results disclosed no significant differences in the virus titer of the two materials tested (Table II).

Discussion. In view of the results obtained in similar experiments with other viruses(2,9), our findings were to be expected, *i.e.*: that topically applied cortisone on rabbit skin enhanced the manifestations of the infection produced by the vaccinia virus. In our experiments, cortisone caused an increase in size of the lesions and an extension of total evolution time, mainly by an increase of the involutive phase. These two effects of cortisone may be explained by its inhibitory properties upon inflammatory and reparative processes. In fact, no differences were observed in either area during the first 4 days, *i.e.*, at the time when important inflammatory changes were not expected to occur. From the fourth day on, cortisone caused progressive increase in the size of the lesions.

As no differences were encountered in the total number of lesions appearing in cortisone-treated areas as compared with those in control areas, it seems that the susceptibility of the cells was not essentially modified. This idea finds further support in the results of the virus titrations on material from treated and control areas. However, differences in amount of virus from the 2 sites might be expected to parallel the differences in size of lesions, and such differences (less than 2-fold) would probably not have been detected by the methods used.

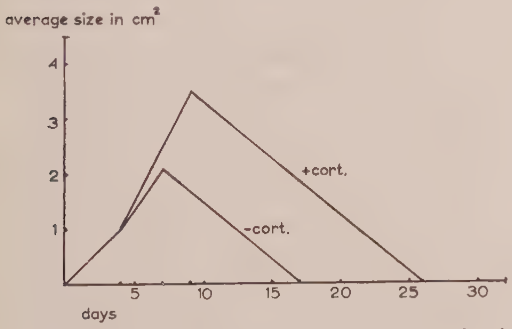

FIG. 1. Evolution time of the lesions and size in areas treated with cortisone and in control areas.

Summary. The effect of topically applied cortisone upon vaccinia virus infection in the rabbit skin was studied. No difference was observed in the number of lesions that appeared in treated as compared with untreated areas, but lesions in cortisone-treated sites attained a greater size than did those in control sites (70% greater area). Also the pathologic process was prolonged by cortisone, from 17 days in control to 27 days in treated areas, namely, by increasing the reparative phase. Comparative titrations, performed in chick embryos and in rhesus monkey kidney cell culture, of the viral contents of lesions from the 2 sites, disclosed no significant difference. These findings may be explained by the inhibitory effect which cortisone exerts upon inflammatory and reparative processes. It seems that cortisone did not essentially modify cell susceptibility to the vaccinia virus.

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Hexosamine and Hydroxyproline Content of Fracture Callus in Normal and Aminoacetonitrile Treated Rats.* (26847)

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Aminoacetonitrile (AAN) administered to adult rats with experimental fractures induces formation of a voluminous fibrocartilaginous callus which ossifies well during the second week of fracture and becomes very osteoporotic during the third week. Connective tissue cells and immature cartilaginous cells are extremely abundant in the callus. Histological and histochemical studies have indicated that the ground substance and collagen fibers are defective(1). We are reporting here studies on the hexosamine and hydroxyproline content of fracture callus of normal and AAN-treated animals obtained 5, 10, 15 and 21 days following the experimental fracture.

Methods. Forty Sprague Dawley male rats weighing from 150 to 155 g were used. Half of the animals received finely ground, laboratory Purina chow and the other half received the same diet containing 0.075% AAN. A fracture in the middle third of both tibiae was produced manually in every rat under anesthesia by bending the leg over the dull edge of a large knife held in a vise. The fractures were not immobilized.

Five experimental and 5 control animals were sacrificed with ether 5, 10, 15 and 21 days following the fracture. Both hind legs were immediately dissected and the fracture callus was collected and placed in dry ice. Also both femurs were obtained, but only the shaft was used, and placed in dry ice. The callus tissue and femurs were pooled and homogenized in acetone in a Virtis blendor, dried and the acetonic powder was used as a starting material for the analysis.

Chemical analysis: The acetonic powder was dried in an oven at 104°C at constant weight and was hydrolyzed in HCl 4N during 15 hours and refluxed at 100°C. After hydrolysis, the solution was dried in a flash evaporator. The residue was dissolved, filtered, and the volume made up with water to 25 ml. This solution was used for the analysis.

The hexosamine was determined with a modification(2) of Boas' method(3). The hydroxyproline was determined according to the method of Neumann and Logan(4) modified by Leach(5).

The hexosamine content in the femoral shaft (including marrow) varied from 5.8 to 7 γ/mg of dried weight and the hydroxyproline content in the femoral shaft varied from 14 to 18 γ/mg in both experimental and control animals. These values did not change markedly throughout the experiment.

Discussion. In the fracture callus of the control animals fed normal laboratory diet an increase of hexosamine was noted which reached its maximum 15 days following fracture. If it is assumed that hexosamine is an index of mucopolysaccharide content of the callus our results agree with the work of Aoike *et al.*(6) who found large increase of chondroitin sulfate in the 2-week-old fracture callus using S^{35} as index of the chondroitin sulfate synthesis. The increase of hexosamine in the callus of the AAN-treated animals is small as compared with the controls. In the 15-day-old callus, the hexosamine of the AAN-treated rats was half that found in the controls and hexosamine decreased very

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TABLE I. Hexosamine and Hydroxyproline Content in Fracture Callus, γ/mg Acetonic Powder Dried at 104°C.

Days after fracture	Hexosamine		Hydroxyproline	
	Exp.	Controls	Exp.	Control
5	20.5	18	30.4	25.3
10	23.4	36.2	24.2	22.9
15	23.8	55.3	27.2	33.4
20	9.3	27.0	16.5	17.5

sharply in the 20-day-old callus. A similar decrease in hexosamine was found in the epiphyseal plates of AAN-treated rabbits by Castellani and Castellani-Bisi(7). Pedrini and Pedrini-Mille also demonstrated that the synthesis of hexosamine in the epiphyseal plates of these animals was greatly decreased in comparison with the controls(8).

On the other hand, no significant differences were found in the hydroxyproline content of the fracture callus of normal and AAN-treated rats.

The values of hexosamine and hydroxyproline in the femoral shafts of normal and AAN-treated rats were similar throughout the experiment.

Summary. The content of hexosamine in the fracture callus of normal rats increased sharply during the first 2 weeks. Much less hexosamine was found in the fracture callus of AAN-treated rats. No significant difference was found in the content of hydroxyproline of the callus of normal and AAN-treated rats.

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Cholinesterases in Serum as Demonstrated by Starch Gel Electrophoresis.*

(26848)

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The demonstration by Alles and Hawes(1) that cholinesterase of red blood cells differs from that of serum has led to our present understanding that 2 types of these enzymes exist, one possessing maximum hydrolytic activity for the 2-carbon acyl group and one for the 4-carbon chain. While other esterases can split some choline esters(2), the property of physostigmine in low concentration to inhibit cholinesterases in a reversible manner(3,4) distinguishes these enzymes from other esterases. In the serum of various species the 2 types of cholinesterases exist in varying proportions(5), predominantly the butyrylcholine-splitting enzyme in the human and the acetylcholine-splitting enzyme in the rabbit. Other authors(6,7) have reported the presence in serum of various butyrylcholine esterases.

In a study of serum esterases by starch gel electrophoresis, a number of bands were obtained, in addition to the expected cholinesterase band, which were active against choline esters and which possessed the property of being inhibited by physostigmine. The number of these bands varied with the species.

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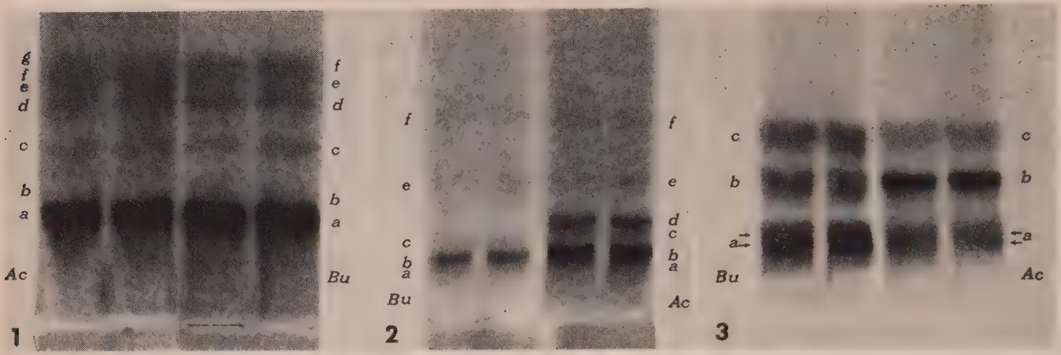

FIG. 1. "Zymograms" of serum cholinesterase. 1. Human. 2. Rat. 3. Cat. (Ac = acetylthiocholine iodide as substrate; Bu = butyrylthiocholine iodide.) Individual bands are lettered.

Methods. Blood was drawn from the human, the cat and the rat by venous or cardiac puncture and after clotting had occurred the serum was obtained by centrifugation. Samples showing hemolysis were rejected. The starch gel was prepared according to Smithies (8) and, after introduction of 50 lambda of serum in each sample slot, the separation was performed by the vertical technic. The cell was placed in a constant temperature incubator at 13°C for 15-18 hours with an applied e.m.f. of 4.5 volts/cm. At completion of the run the block was cut into halves and then into strips along the plane of migration. Each strip embraced 2 sample slots and was placed in a glass tray and immersed in the appropriate incubating medium. In inhibition experiments the gels were pre-treated with the inhibitor at room temperature for 30-60 minutes in a solution identical to the final incubating medium save that the substrate was lacking. This pre-treatment was followed by incubation in the full substrate plus inhibitor. All incubations, with or without the presence of inhibitors, were for 4 hours at 37°C.

The substrate solutions contained acetylthiocholine iodide or butyrylthiocholine iodide and were identical to those devised by Gomori(9) except that 7.5 g % polyvinylpyrrolidone was substituted for saturated sodium sulfate. Inhibitors used were physostigmine sulfate (1×10^{-5} to 1×10^{-4} M), DFP (di*iso*propyl fluorophosphonate) (1×10^{-5} M), 62C47 [1:5-(4-trimethylammoniumphenyl) pentan-3-one diiodide] (5×10^{-5} to 1×10^{-4} M), and mytelase [N′, N′, -bis-(2-diethylaminoethyl) oxamide bis-2-chlorobenzyl chloride] (10^{-6} M). The bands of enzyme reaction product were visualized by immersion of the gel strips in 5% $(NH_4)_2S$. A dark brown or black zone of copper sulfide represented the site of cholinesterase activity.

Results. The results obtained with serum of human, rat and cat are shown in Fig. 1. These are unretouched photographs. It can be seen readily that the sera of the 3 species show the presence of a number of stained areas with both acetylthiocholine iodide (AcThCh) and butyrylthiocholine iodide (BuThCh). In human serum there is a prominent band of activity (band A) against both substrates near the origin and, additionally, 5 lighter staining zones (B to F) can be discerned. Against AcThCh another discrete area of staining is demonstrable at G. In addition to these well-defined zones of staining, a rather diffuse pale area of coloration occurs proximal to A and merging with it. Employment of physostigmine (1×10^{-5} M) as an inhibitor results in complete disappearance of bands B to G, but band A is only partially inhibited (about 50% as judged visually). With 10^{-4} M physostigmine inhibition of band A is almost, but not quite, complete. Use of DFP 10^{-5} M results in complete abolition of all enzymatic activity. Thus it would seem that part of the hydrolysis of AcThCh and BuThCh is accomplished by an esterase similar to Aldridge's B- esterase(10) in respect to its relative insensitivity to eserine and its sensitivity to DFP. Yet B- esterase is believed to be present only in erythrocytes in the human, being absent from plasma(11).

Neither 62C47 nor mytelase, both acetylcholinesterase inhibitors, caused any inhibition and this suggests that none of the activity observed was due to this enzyme.

In rat serum, hydrolysis of AcThCh resulted in 6 bands, 2 of them prominent, while there were only 5 bands of activity against BuThCh, the prominent band D being absent with this substrate. A number of further distinctions between human and rat serum appear when enzymatic activity is studied with the aid of inhibitors. In the rat all activity against both substrates is abolished by physostigmine 10^{-5} M, while 62C47 10^{-4} M abolishes band D. No inhibition occurs with mytelase. In the case of cat serum, both substrates yield 3 rather diffuse zones of staining. In the zone nearest the origin 2 distinct dark bands may be discerned (arrows) against the diffuse background coloration. BuThCh gives approximately equal staining in zones A, B and C but, in the case of AcThCh, a discrete dark band appears in zone B. All the enzyme activity of cat serum was eliminated by physostigmine 10^{-5} M. DFP 10^{-5} M completely inhibited zones A and C only.

Discussion. The results described indicate that the hydrolysis of choline esters in serum is more complex than has been realized. Thus, in the rat, 6 eserine-sensitive enzymatically active zones can be visualized with AcThCh as substrate. In the human, an even larger number of cholinesterases, but with different sensitivities to inhibitors, is demonstrable. Whether the various zones delineated represent specific molecular species of enzymes, or whether they represent one or several enzymes adsorbed onto inert proteins and migrating with the latter is the object of further study by quantitative methods in this laboratory. Others have adduced evidence, admittedly not final, in favor of the specific identity of the enzymatic activities demonstrated by "zymogram" technics(12), and the results described here (variations of staining pattern with each substrate and variations in sensitivity to the different inhibitors employed) would uphold such a view.

Summary. As many as 7 bands of enzymatic activity against choline esters are demonstrable in human, rat and cat sera by starch gel electrophoresis. The evidence suggests that these bands derive from distinct molecular species of enzyme. However, possible influences on "zymogram" patterns of enzyme adsorption by inert protein require investigation.

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Connective Tissue IV. Effect of Age upon Dermal Chemical Response to Adrenal Hormones.* (26849)

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The adrenal gland plays a major role in hormonal control of the connective tissue(1). Considerable information is available concerning the effect of cortisone upon the histochemistry of this tissue(2,3). Chemically, Layton(4) has shown that rate of synthesis of chondroitin sulfate was inhibited by massive doses of cortisone, while Sobel *et al.*(5,6) have reported that large doses of hormone resulted in a decrease in dermal hexosamine, but had no effect upon dermal collagen content.

Using a sequential extraction procedure for chemical dissection of connective tissue into ground substance, neutral and acid soluble collagen, and insoluble collagen(7), this paper describes the effect of both deoxycorticosteroid (Doca) and hydrocortisone (cortisol) administration upon the chemistry of rat skin. These results were obtained using rats of 3 different weights; 150, 270 and 370 g representing young, mature and aged animals respectively(8).

Materials and methods. Three groups of 18 male Sprague-Dawley rats each weighing an average of 150, 270 and 370 g were employed. Six animals from each group were injected daily for 7 days with 3 mg/kg body weight subcutaneously of cortisol (Cortril, C. Pfizer & Co.); another 6 animals from each weight group were injected similarly with 3 mg/kg of Doca (Organon); the remaining animals were used as normal controls. All animals were sacrificed on the seventh day and about 2 g of abdominal skin from each animal were shaven, removed, dissected free of adhering fat, muscle and fascia, and minced. Aliquots from each tissue sample (0.5 g) were hydrolysed in 4N HCl for 8 hours at 100°C. The cleaned skin remaining from each group of 6 rats was pooled and sequentially extracted in duplicate with 0.15M NaCl, 0.5M NaCl and 0.50M citrate buffer (pH 3.6) in the cold as described previously (7). These extracts were also hydrolysed in 4N HCl. The hydrolysates of both extracts and whole skin were analysed in triplicate for their hydroxyproline(9), hexosamine(10) and nitrogen(11) concentration as a measure of the collagen(12), ground substance (13) and protein content of the skin, respectively. These results were expressed as the ratio of the mean hexosamine of hydroxyproline content (in μmoles) per mmole of total dermal nitrogen of each group of animals, to minimize the changes in dermal water and fat produced by hormone administration.

Experimental and results. Analyses of the saline soluble components of rat skin 7 days after starting either daily cortisol or daily Doca administration are shown in Table I with the results obtained from normal animals of a similar weight. These results indicate that both hormones produce a decrease in ground substance (ratio of hexosamine to nitrogen in the 0.1M NaCl extract) except in the aged animals. Isotonic soluble hydroxyproline/nitrogen was increased with Doca administration only in the aged animal, while both hormones produced decreases of this material in younger animals. The increase in hydroxyproline/nitrogen ratio of the isotonic extracts of the skin of aged animals with Doca was the only difference in the dermal chemical effects of this hormone as compared with cortisol.

Both the hydroxyproline and hexosamine to nitrogen ratios in the 0.5M NaCl extracts were decreased below normal with both hormones in animals of all 3 weights studied.

Analyses of both the citrate buffer soluble and the insoluble components of the skin of rats after either cortisol or Doca administration are shown in Table II. These results show that only with the older animals did

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TABLE I. Effect of Age upon Dermal Chemical Response to Adrenal Hormones (Saline Soluble Components).

Wt, g		0.15M NaCl		0.50M NaCl	
		Hex/N†	Hypro/N†	Hex/N†	Hypro/N†
150	Normal	1.90	1.90	.74	3.51
	Cortisol	.87*	1.10*	.61	2.90
	Doca	.50*	.85*	.25*	1.23*
270	Normal	.96	1.05	.37	4.00
	Cortisol	.47*	.20*	.27*	1.00*
	Doca	.48*	.34*	.33	2.60*
370	Normal	.43	.70	.38	2.51
	Cortisol	.50*	.38*	.26*	.38*
	Doca	.53*	1.00*	.29*	1.06*

* Significantly different from normal ($p <.05$).

† Hex = μmoles hexosamine; Hypro = μmoles hydroxyproline; N = mmoles nitrogen.

both hormones result in appearance of citrate soluble hexosamine. Animals of all 3 weights demonstrated an increase in citrate soluble collagen with cortisol. Doca was less effective in this regard than cortisol, and with increasing body weight, this hormone became even less active than cortisol until, in the aged animal, Doca resulted in a decrease in dermal concentration of citrate soluble collagen.

The insoluble hexosamine content of the skin from young animals was increased only with administration of Doca. With older animals, both hormones resulted in an increase in this material in the skin. Insoluble collagen was decreased by both hormones in all 3 weight-ranges of animals studied.

The percent changes from normal with respect to total dermal hexosamine and hydroxyproline to nitrogen ratios induced by cortisol or Doca in young (150 g), mature (270 g) and aged (370 g) animals are shown in Table III. These data indicate that although both hormones produced qualitatively similar changes in dermal chemistry, these changes were quantitatively different. Further, Doca and cortisol produced quantitatively different effects upon soluble collagen concentration only in the skin of aged rats.

With increasing age, the total hexosamine to nitrogen ratio in rat skin responded differently to administration of both cortisol and Doca. Total dermal collagen concentration was increasingly depressed from normal with increasing age in response to both cortisol and Doca administration. In either young or mature animals, both hormones decreased the dermal concentration of ground substance. In the aged animal, however, both cortisol and Doca resulted in an increase in ground substance.

Except for the obverse effect of Doca in the aged animal, both hormones decreased the neutral soluble collagen and increased the

TABLE II. Effect of Age upon the Dermal Chemical Response to Adrenal Hormones (Citrate Soluble and Insoluble Components).

Wt, g		0.50 citrate		Insoluble	
		Hex/N†	Hypro/N†	Hex/N†	Hypro/N†
150	Normal	0	2.7	0	28.5
	Cortisol	0	4.3*	0	21.0*
	Doca	0	4.0*	.83*	24.5*
270	Normal	0	1.6	.60	41.5
	Cortisol	0	5.1*	1.30*	28.0*
	Doca	.30*	4.2*	1.00*	28.1*
370	Normal	0	3.4	.65	47.0
	Cortisol	.33*	5.0*	1.00*	26.1*
	Doca	.27*	1.7*	1.08*	30.0*

* Significantly different from normal ($p <.05$).

† Hex = μmoles hexosamine; Hypro = μmoles hydroxyproline; N = mmoles nitrogen.

TABLE III. Percent Changes from Normal in Dermal Chemistry with Hormone Administration in Young, Mature and Aged Rats.

		Hormones					
		Cortisol			Doca		
		Young	Mature	Aged	Young	Mature	Aged
Total:	Hex/N	−42	0*	+80*	−39	0*	+40*†
	Hypro/N	−20	−29*	−41*	−16	−26*	−36*
	.15M NaCl Hex/N	−52	−51	+25*	−73†	−50*	+25*
Soluble Hypro/N:	.5M NaCl	−42	−80*	−46	−55†	−67*†	+43*†
	.5M citrate	+59	+210*	+47*	+48†	+163*†	−50*†
	Insoluble Hypro/N	−26	−33	−45*	−14†	−33*	−36

* Significantly different from effect in the previous age group ($p < .05$).
† Significantly different from effect produced by cortisol in a similar age group ($p < .05$).

citrate soluble collagen concentration of the skin from animals of all 3 ages. Finally, both hormones were increasingly effective with age, decreasing the dermal concentration of insoluble collagen.

Discussion. With the exception of the aged animal, both cortisol and Doca produced remarkably similar effects upon the chemistry of rat skin. In view of the primary mineralocorticoid effect of Doca, the similarity of effects produced by cortisol and Doca suggests either a hitherto unknown property of Doca or that changes in electrolytes and water eventually have profound consequences upon the metabolism of the connective tissue. Since the data reported above were based on nitrogen content of the skin, it would seem unlikely that these changes in dermal chemistry with cortisol or Doca would result directly from changes in tissue water content.

The primary effect of adrenal hormones upon the chemistry of the skin is to decrease total and insoluble dermal collagen concentration. Changes in total hexosamine and ground substance with hormone administration seem to depend largely upon the age of the animal. Presumably this represents either the synergistic or antagonistic activities of various sex and growth hormones with adrenal hormones acting upon the connective tissue or changes with ageing in responsiveness of the connective tissue itself to these hormones.

Sobel *et al.*(5,6) found that cortisone was without effect upon collagen content of rat skin. In young animals, such as this group employed in their studies, collagen concentration decreased 20%. Either these workers could not analytically determine a change in dermal collagen concentration of this magnitude, or the large doses of drug employed by them over a long period of time resulted in a different response to cortisone. Our data and those reported by them with respect to both dermal hexosamine loss and decrease in hexosamine to collagen ratios with cortisol administration are essentially in agreement.

Both Doca and cortisol resulted in a decrease in neutral soluble and insoluble collagen and an increase in citrate soluble collagen in young or mature animals only. Quantitatively these changes in dermal collagen are remarkably similar to those produced in uninjured skin distant from the site of local injury(11,14). Perhaps these changes with local inflammation proceeded *via* the trauma of injury and consequent release of minute amounts of adrenal hormones. This work on inflammation was performed only on mature animals, and no changes in total hexosamine with local injury were either found(14) or should be expected from the data on Table III. If adrenal hormonal mechanisms are involved in this "distant dermal collagen response to local inflammation"(14,15), then inflammation in young or aged rats should result in either a loss or an increase in hexosamine concentration of the uninjured skin (Table III) respectively.

Summary. Both cortisol and Doca similarly affect the chemistry of the connective tissue in that they result in a decrease in total and insoluble collagen concentration of rat skin. The effects of these hormones upon rat skin are profoundly altered by the age

of the animal. The aged rat demonstrates a considerably different dermal chemical response to cortisol and particularly Doca than does either the young or the mature animal.

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Relation of Selenium, Vitamin E and Other Factors to Muscular Dystrophy in the Rabbit. (26850)

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Among various species there are widely differing effects of a dietary lack of Vit. E, the most common manifestation of which is muscular degeneration. Morgulis and Spencer (10) found evidence for a factor in alfalfa or whole wheat germ which was necessary for preventing muscular dystrophy in the rabbit in addition to a fat-soluble factor (Vit. E). MacKenzie and McCollum(9) reported that Vit. E alone was capable of preventing this deficiency syndrome. A lack of many other nutritional factors has also been implicated in producing muscular dystrophy; among them are choline(6,8), potassium(7), phosphorus(13), Vit. C(4), Vit. B_6(3), and selenium(11,12). Other factors have been shown to increase the severity, such as iodide (1) and even exercise. Differences in these factors could explain the contradictory evidence presented by Morgulis and Spencer (10) and by MacKenzie and McCollum(9).

Methods. Two experiments were independently conducted at the Ontario Agricultural and Veterinary College and at Cornell University. The objectives of each were to determine the effect of alpha tocopherol and selenium upon incidence of muscular dystrophy in the rabbit. In addition, the Cornell experiment was designed to include diets to which natural feedstuffs had been added. These feeds were chosen because muscular dystrophy often occurs in lambs when these feeds are fed(5,12). Diets used in the experiments are shown in Table I.

Results and discussion. An alopecia was noticed in all of the rabbits fed diets containing no "natural feed." A loss of the long guard hairs began along the ventral part of the abdomen about one week after the rabbits were placed on the experimental diets. Since the rabbits were housed in groups of 5 it was not possible to determine the extent of hair chewing that might have taken place.

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TABLE I. Basal Diets Used in the 2 Experiments.

Ingredients	I (Ontario)	II (Cornell)
	(%)	
Torula yeast	40.0	39.0
Dextrose*	40.0	39.0
Lard†	10.0	10.0
Bulk material‡	10.0	10.0
NaCl	1.0	1.0
$CaCO_3$	.5	—
$Ca_3(PO_4)_2$	.5	1.0
Vitamins	— §	— ‖

* Cerelose 2001, Corn Products Refining Co.

† Stripped lard, furnished by Distillation Products Industries, Rochester, N. Y.

‡ Ontario experiment — Cellulose (Alpha-cel), Nutritional Biochemical Corp., Cleveland, O.
Cornell experiment — Cellophane (Jelflake) ground to a particle size that would pass a ⅛ inch mesh.

§ Vit. A, 1200 I.U./kg feed; Vit. D, 170 I.U./kg feed; Menadione, 2 mg/kg feed.

‖ Vit. A, 400 I.U./kg of diet, Nopcay 20, Nopco Chemical Co.; Vit. D, 165 I.U./kg of diet, Dupont Delsterol 3000-D.

Nevertheless, since the entire body covering was affected, it is believed that most of the hair loss was due to dietary inadequacies.

At death, or termination of experiment, all rabbits were examined for gross anomalies, and skeletal and cardiac muscle samples were subjected to histopathologic study. These data as well as average time of survival and body weight changes are presented in Table II.

Neither selenium nor Vit. E singly or in combination was effective in preventing muscular dystrophy at the levels fed. In the Ontario experiment the level of Vit. E was considerably above that which is generally considered the daily requirement. In the Cornell experiment, estimates of daily food consumption place the intake of Vit. E approximately at the requirement of the rabbit (0.30 I.U./kg body weight per day). The selenium content of the basal ration (Cornell), determined by a neutron activation procedure performed by Oak Ridge National Laboratory, Tennessee, was found to contain 0.02 ppm selenium. Thus, in diets where Na_2SeO_3 was added there was a total content of 1.02 ppm selenium. A 20% level of wheat bran or linseed oil meal in the diet did not prevent the condition entirely, although one and two rabbits survived the experiment on these 2 diets, respectively. Two rabbits survived the experiment on the diet containing 40% of cooked kidney beans; however, these rabbits showed microscopic lesions of muscular dystrophy.

Evidence exists(2,5) for a factor in some feeds which may enhance muscular dystrophy in the lamb. Torula yeast is commonly used in diets that will produce certain associated conditions in various species; *i.e.*, die-

TABLE II. Effects of Selenium, Vitamin E and Several Natural Feeds on Muscular Dystrophy, Survival and Body Weight Changes in Rabbits.

Treatment	No. of rabbits	Avg days of survival	Body wt (g)		No. surviving test period	No. showing muscular dystrophy	
			Initial	Final		Clinical signs	Lesions upon necropsy
		Ontario Exp.					
Basal	5	44	510	567	0		5
" + vit. E*	5	43	472	598	1		3
" + selenium (1 ppm)	5	45	530	629	0		5
		Cornell Exp.					
Basal	5	26	1360	1205	0	5	3‡
" + vit. E†	5	32	1324	1122	0	5	4‡
" + selenium (1 ppm)	5	28	1330	1177	0	5	3‡
" + linseed oil meal (20%)	5	52	1403	1642	2	3	5
" + wheat bran (20%)	5	45	1311	1287	1	4	5
" + cooked kidney beans (40%)	5	48	1368	1721	2	3	4‡
" + vit. E† + selenium	5	31	1306	1118	0	5	4‡

* 3.4 mg of dl-alpha tocopheryl acetate/kg of body wt daily.

† 22 I.U. of dl-alpha tocopheryl acetate/kg of diet.

‡ Complete data not obtained due to postmortem changes.

tary liver necrosis in rats(14) and exudative diathesis in chicks(15). It is therefore possible that the basal diet was deficient in certain factors involved in prevention of muscular dystrophy (Vit. E, selenium and perhaps methionine and cystine) as well as containing inhibitors or antagonists that may enhance the occurrence of muscular dystrophy.

Summary. Rabbits maintained on a torula yeast, Vit. E deficient diet developed a severe and rapidly progressing muscular dystrophy. This condition was not prevented by addition to the diet of 1 ppm selenium or of supplements of dl-alpha tocopheryl acetate alone or in combination. Additions of natural feedstuffs (wheat bran, linseed oil meal or kidney beans) to the semi-purified diet did not fully prevent the condition, although they did lessen the severity of the lesion and increase the average number of days of survival indicating deficiencies other than selenium and Vit. E were involved in the particular experimental diets used. A marked alopecia was observed in all animals fed diets not containing a natural feedstuff.

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Immune Response to Actinophage in the Mouse.* (26851)

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Recently about 25 actinophages virulent for members of the genera Streptomyces, Nocardia, Micromonospora, Actinoplanes and Mycobacteria have been isolated and characterized with respect to host range(1). The conditions for growth of these bacterial viruses, their antigenic relationships to one another and their distribution in nature have been partially determined(2). Moreover, actinophage plaque-forming units can be enumerated easily, quickly and accurately(3). Therefore, actinophages, as well as bacteriophages of *Escherichia coli*(4), possess many desirable properties required of an antigen used in the study of onset of antibody formation. This report is concerned with the antigenicity of actinophage and uptake and retention of the viral particles by organs of the mouse.

Uptake and retention of actinophage. Inbred, male, adult, Bagg albino mice (a sub-

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TABLE I. Appearance of Actinophage in Organs after Intraperitoneal Presentation.

Organ	No. samples	Log No. plaque-forming units/g tissue After 5 min.	 After 3 hr
Spleen	14	8.9	>9.0
Lung	21	6.3	8.4
Testis	14	7.8	8.5
Brain	4	4.2	6.2
Liver	8	7.6	>8.0
Kidney	10	7.4	7.9
Whole blood	17	4.3	4.8
Serum		4.2*	4.6*
Buffy coat		2.7*	3.2*
Washed red cells		3.3*	2.9*

Balb mice were inj. intraper. with 10^{10} actinophage MSP8. Subsequently, organs were excised, washed, homogenized and assayed for viable virus. Data are presented as geometric means.

* Virus content of fraction derived from 1 g of whole blood.

line of BALB/Sy) were injected intraperitoneally with 10^{10} plaque-forming units of actinophage MSP8. Blood samples were collected and lungs, liver, brain, kidney, spleen and testis were removed 5 and 180 minutes after injection. Serum was obtained after centrifugation in capillary tubes. Blood was mixed with an inoculative suspension of the virus-host *Streptomyces venezuelae* S13 in molten peptone-yeast-extract agar at 48°C and poured into petri plates containing a basal layer of the same medium(3). The excised organs were washed in peptone-yeast-extract broth, dispersed with a tissue grinder, diluted appropriately, mixed with molten, seeded overlay medium and dispensed into petri plates. Viable actinophage was recovered from all of the organs tested (Table I). Viral content of blood collected from the retro-orbital plexus, tail and toes of the fore and hind limbs was the same. Actinophage appeared in the blood immediately after injection; a sample taken as quickly as possible (less than 15 seconds) contained in excess of 5×10^3 particles/ml. It should be noted that the virus was in the serum; the cellular fraction of the blood was essentially virus-free (Table I) Phage could not be isolated regularly from the blood 2 days after presentation of antigen, but was detected in lung, liver and kidney for 6 days, and in testis and spleen after 10 days (Fig. 1).

When 10^7 or fewer virus particles were administered, they were not recovered in the blood but were found in the lung and testis. The lung did not retain as many phage particles or hold them as long as the testis (Table II).

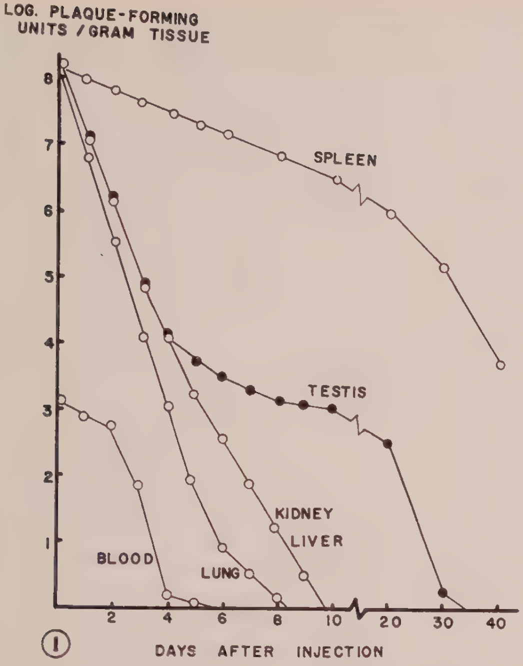
Antibody production. Mice injected with 10^{10} MSP8 were bled daily for 4 days and the titer of neutralizing antibody in each serum determined by the method of Lennox *et al.*(5). Specificity of the reaction was established by including bacteriophage T2, which lysed *Escherichia coli* but not *S. venezuelae,* in the assay mixture. Plaque-forming capability of MSP8 was reduced markedly by serum collected 4 days after presentation of antigen, but T2 was unaffected. Substantial antibody activity was detected in the serum taken 48 hours after antigenic stimulation (Fig. 2). Antibody production was influenced by the amount of antigen administered. Mice challenged with fewer than 10^7 MSP8 did not produce measurable antibody. The optimal concentration for provocation of neutralizing activity fell between 10^{10} and 5×10^{10}; doses of 10^{11} and 2×10^{11} evoked no better or an inferior response (Table III).

Discussion. Many years ago, Buxton demonstrated that living typhoid bacilli, introduced into the peritoneum of the rabbit, enter the general circulation almost immedi-

TABLE II. Effect of Actinophage Dose on Virus Uptake.

No. virus injected	Sample taken, min. after inj.	Log No. plaque-forming units/g tissue Blood	 Lungs	 Testis
10^{10}	5	4.4	6.4	8.0
”	180	4.3	8.4	8.7
10^{9}	5	2.4	5.3	7.3
”	180	3.5	7.2	7.8
10^{8}	5	1.7	4.6	5.9
”	180	3.0	5.4	6.8
10^{7}	5	<1.3	<3.0	5.1
”	180	<1.3	5.0	5.3
10^{6}	5	<1.3	<3.0	3.8
”	180	<1.3	<3.0	4.9

Balb mice were inj. intraper. with actinophage MSP8. Subsequently organs were excised, washed, homogenized and assayed for viable virus. Data are presented as geometric means of samples taken from 15 mice.



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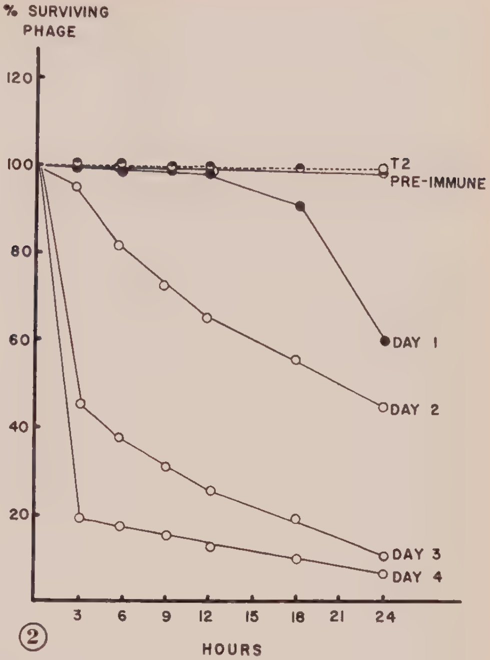

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FIG. 1. Retention of viable actinophage in organs of Balb mice inj. intraper. with 10^{10} plaque-forming units. Each point is the geometric mean of samples from 2-15 mice (mode = 5).

FIG. 2. Onset of antibody production. Balb mice were inj. with 10^{10} MSP8, bled daily for 4 days and neutralizing activity of serum diluted 1:15 determined with respect to MSP8 and *E. coli*-phage T2. Phage + serum mixtures were incubated at 30°C. Each point is the arithmetic mean of samples from 25 mice.

ately and concentrate in the liver and spleen subsequently(6). Similarly, actinophage administered intraperitoneally is found in the circulatory system in less than 15 seconds and is distributed throughout the body of the mouse within 5 minutes. Route of entry of the bacterial virus has not been determined, but may be through the peritoneal capillary circulation or possibly through the lymphatic vessels. Viable actinophage is retained in the animal, in particular by the spleen, for more than 6 weeks. Prolonged persistence of other antigens(7,8) is now well established; however its significance for genesis and maintenance of antibody is not apparent. It is interesting that 10^7 phage particles were sufficient to invoke measurable antibody production. This amount of actinophage contains less than 0.01 μg protein, or assuming an average molecular weight of 10^5 for protein, fewer than 6×10^{10} molecules. Bacteriophage contains at least 6 distinct components(9), most of which probably are not concerned with neutralization. If we assume that the mouse contains 10^{10} reticulo-endothelial cells, then there is only one "neutralizing antigen" molecule per RE cell. In our experiments, it is possible that many

TABLE III. Effect of Actinophage Dose on Antibody Production.

No. MSP8 inj.	% survival of virus after incubation with 1:20 serum			
	2-day sample		4-day sample	
	MSP8	T2	MSP8	T2
2×10^{11}	76	100	9	99
10^{11}	47	101	5	100
4×10^{10}	32	100	2	98
10^{10}	61	98	6	98
4×10^{9}	83	98	22	97
10^{9}	88	99	57	98
10^{8}	98	101	63	100
10^{7}	104	100	71	99
10^{6}	101	100	96	98

Balb mice were inj. intraper. with actinophage MSP8 and bled on day 2 and 4. Serum was heated 56°C for 30 min. and mixed with actinophage MSP8 and *E. coli*-phage T2. The virus + antiserum mixture was incubated for 24 hr at 30°C, diluted and plated with *Streptomyces venezuelae* S13 or *Escherichia coli* B. Data are presented as arithmetic means of samples taken from 15 mice.

immunologically competent cells were not exposed to antigen. Equally significant, detectable specific antibody is made within 24 hours after antigenic stimulation. Similarly, Lennox and Avegno (private communication) has found neutralizing antibody 24 hr after antigenic stimulation of rabbits with coliphage T2. As measurements become more sensitive, it may be possible to demonstrate antibody production immediately after presentation of antigen. It should be emphasized that this possibility does not disprove the inductive hypothesis for genesis of antibody. The enzyme-forming system for synthesis of adaptive (induced or repressed) enzyme is present in the unadapted cell. In systems where the inducer is not a substrate, enzymic activity increases almost instantaneously and linearly after addition of inducer(10). Our results do not support preferentially the clonal selection, natural selection or inductive theory of antibody synthesis. This study does establish that the actinophage-neutralizing antibody system in the mouse is an excellent tool for determination of the minimum time and antigenic dose necessary for antibody synthesis.

Summary. Actinophage injected intraperitoneally into inbred Balb mice appeared almost instantaneously in blood, brain, liver, kidney, spleen, lung and testis. Viable virus was isolated from the blood for 2 days following challenge, from lung, liver and kidney for 6 days, and from testis and spleen for more than 10 days. Mice produced neutralizing antibody within 48 hours after antigenic stimulation. Antibody production was elicited by as few as 10^7 actinophage particles.

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Excision of Infarcts of the Left Ventricle and Replacement by Teflon Prostheses. (26852)

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Murray(1) and Bailey(2) have demonstrated that excision of a myocardial infarct in the dog can be successfully performed. However, the latter showed that the procedure usually resulted in a reduction in left ventricular capacity and cardiac failure. To obviate this, we undertook a series of experiments to determine the practicability of replacement of the excised myocardium with a teflon prosthesis. The results of 10 experiments are reported here.

Procedure. We attempted first to develop a technic which would have an acceptably low morbidity and mortality. In a pilot series of experiments, operations on the left ventricle were performed under cardiopulmonary bypass and aortic occlusion. Later, we added systemic, and then selective (cardiac) hypothermia, but none of these animals survived. It was apparent that a simpler technic, avoiding extracorporeal circulation and hypothermia must be employed. The following procedure was developed and has been performed successfully in 10 consecutive dogs.

The animal is anesthetized with intravenous sodium pentobarbital, a cuffed endotracheal tube inserted and connected to an automatic respirator (neophore). The pleura is entered through the left fifth interspace, the pericardium is opened widely in front of the phrenic nerve, and the heart delivered. The lowest major trunk of the anterior descending branch of the left coronary artery is ligated, producing an area of cyanosis and flaccidity for 3 or 4 sq cm over the anterior wall and apex of the left ventricle.

A strip of teflon felt one-eighth inch in thickness and one-quarter inch in width is placed on either side of the infarcted area, leaving one or two ml of normal muscle medial to the strips. Four horizontal matress sutures of 2-0 silk are then passed through one strip of teflon, the left ventricular wall, through the opposite teflon strip and then back. To avoid inclusion of important valvular structures, these sutures are placed so as barely to enter the cavity of the heart. When they are tied snugly over the teflon strips, the infarcted segment is excluded from the cavity of the ventricle. The necrotic muscle is then excised and a tailored teflon patch one-eighth inch in thickness secured with continuous and interrupted 2-0 silk sutures to the margins of the defect. Usually, the teflon strips on either side are also incorporated in the suture line. The mattress sutures are now removed, and the heart resumes its previous contour and size. Usually there is some leakage of blood across the graft, but this is controlled by gentle pressure for several minutes. In one animal, (#7), teflon felt of 1/16th inch thickness was used. Bleeding across this patch was excessive, but was controlled after 10 minutes of pressure. Routinely, the estimated amount of blood lost during the operation was replaced by transfusion.

Results. All animals survived the procedure, the first having been operated upon almost 3 months ago. To date, there is no evidence of infection, systemic embolization, congestive heart failure or rupture of the graft. One animal was sacrificed one week postoperatively. The graft, of 1/16th inch teflon felt, was securely attached to the heart muscle, markedly adherent externally to thickened, inflamed pericardium, and covered internally by a layer of fibrin. It had very effectively sealed the defect in the myocardium, and was already firmly incorporated into the left ventricular wall. Soft clot lay in a small intramural chamber which communicated with the main cavity of the left ventricle by the endocardial defect, which was 1.5 cm in diameter.

Discussion. Murray(1) observed that when a major branch proximal to the 2 or 3 large terminal branches of the left coronary artery was ligated, there was cyanosis, dilatation, lack of contraction, and paradoxic systole over the site of distribution of the vessel. Because the contractile force of the left ventricle is partially dissipated by this paradoxically pulsating chamber, there is a reduction in stroke volume, and cardiac output falls. He therefore suggested that excision of acute myocardial infarcts and primary closure of the defect by direct suture might be of benefit. In 25 dogs subjected to this procedure, blood pressure and cardiac output rose after the infarct was excised, and many of these animals were alive one year later. Bailey(2) repeated these experiments. Six of the 25 dogs in his series died of ventricular fibrillation, 2 of bleeding, and one of empyema; 13 others died early because of congestive heart failure, and 3 were sacrificed. Autopsy revealed that there was a significant reduction in capacity of the left ventricle, and he felt that this accounted for the congestive heart failure which caused death in approximately half of the animals.

If irreversible shock or congestive heart failure in patients following myocardial infarction is due to the presence of a non-contracting, paradoxically pulsating expansion chamber made up of necrotic, atonic muscle and scar tissue, consideration should be given to its excision. This, of course, would require operation under cardiopulmonary bypass. It occurred to one of us (A.D.) that to avoid serious reduction in left ventricular capacity following excision of an infarct, with resultant congestive heart failure, it should be replaced by an inert plastic prosthesis of equivalent size. In other words, no pulsation is probably preferable to paradoxic pulsation, a concept supported by the long and asymptomatic survival of human beings with large, calcified aneurysms of the left ventricle.

Teflon felt of one-eighth inch thickness was selected because this material is soft, non-brittle, inert, durable, non-wettable, resistant to infection and easy to suture. Such prostheses have been demonstrated to act as an inert matrix for deposition of a thin layer of fibrin over the surface in contact with the blood, and for fibrous tissue which grows through the graft itself. Because of low tissue reactivity, the healing time of the implant should be rapid. We may find that woven teflon, similar to that used in synthetic grafts or as an outflow patch in the right ventricle, is preferable. We believe, however, that the felt will be well tolerated, and will eventually be incorporated in the wall of the left ventricle. This will be determined when chronic survivors are sacrificed.

To date, use of a prosthesis has not been attended by complications reported by other investigators after left ventriculotomy. For example, Carter(3) reported that 2 of 25 dogs died of delayed rupture following left ventriculotomy. In another series(4) 3 of 22 dogs with excision of myocardium, and 3 with linear incisions into the left ventricle, died of rupture. In a third group of dogs reported by Dillon(5), portions of the left ventricle were excised in 22; 6 died of rupture, and only 6 were long-time survivors. Wounds of the left ventricle disrupt because of strangulation of the heart muscle by the suture material, almost certainly because of tension on the line of sutures. So far, none of our animals has died of secondary rupture or congestive heart failure. We believe, therefore, that by using the prosthesis, such complications will probably be avoided.

In the one animal we have sacrificed, some clot lay in the chamber formed by excision of the full thickness of the left ventricular wall, including the endocardium. We believe this is caused by the fact that the graft is not sutured to the endocardium, but rather to overlying muscle and epicardium, leaving an intramural diverticulum behind. In the future, attempts will be made to leave the endocardium intact, or if exposed, to suture the felt to this as well.

Summary. Ten mongrel dogs have been subjected to experimental ligation of major branches of the anterior descending trunk of the left coronary artery. The infarcted areas have been removed by a closed technic and replaced by a teflon felt prostheses. All of the animals have survived. Two were sacrificed one week following the operation, while eight others are now being followed for periods of up to four months. They will be sacrificed at the end of a year.

To this time, the implanted material appears to be well tolerated by the myocardium.

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Immunobiological Identification of Human Chorionic Gonadotropin.*† (26853)

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Human chorionic gonadotropin (HCG) has been difficult to obtain in an immunochemically pure form. Early workers using crude preparations were unable to determine whether this mucoprotein was capable of eliciting the formation of specific precipitating and neutralizing antibodies(1). That antigenic impurities are present has been clearly shown by Got(2) who prepared rabbit antiserum to HCG assaying 1500 I.U./mg and demonstrated by immunoelectrophoresis the presence of 8 antigen-antibody precipitin bands. Extensive purification resulted in a preparation which assayed 12,000 I.U./mg and gave a single band following immunoelectrophoresis with the same antiserum. McKean(3) developed an antiserum to a purified preparation of HCG assaying 8,000-9,000 I.U./mg and described its use in detecting HCG in urine of pregnant women.

In spite of apparent specificity of antibodies to HCG, and especially of antibodies to anterior pituitary hormones(4), it has been suggested that these "hormone specific" antibodies may be directed against carrier proteins differing in urine and serum(5). This report describes the preparation and characterization of an antiserum against HCG, and demonstrates the ability of this antiserum to identify HCG of both benign and malignant trophoblastic origin and to neutralize the biological activity of HCG in serum or urine.

Materials and methods. Immunization procedure. Adult rabbits were given subcutaneous injections of 10,000 to 20,000 I.U. of commercial human chorionic gonadotropin|| (HCG-U) emulsified in incomplete Freund's adjuvants. This procedure was repeated 5 times at intervals of 1 to 3 weeks. Thirteen days after the last injection, the animals were bled and the antisera pooled.

Antigens. Pregnant human serum (PHS) and urine (PHU) were collected between the 50th and 90th days of gestation. Normal human serum (NHS) was obtained from Microbiological Associates and normal human urine (NHU) from young adult males. Sera were also obtained from golden hamsters heterotransplanted with choriocarcinomas (Pitt 89 and Pitt 100) and an embryonal carcinoma (Deac 3), all of human testicular origin. Pitt 89 and Pitt 100 have consistently secreted chorionic gonadotropin, whereas no evidence of gonadotropic activity has been detectable in the serum from hamsters bearing Deac 3(6).

The sera were concentrated to enhance gonadotropic activity according to modifications of the procedures of Delfs(7) and Landgrebe(8). Fifty milliliters of each serum were extracted 5 times with ether, precipitated with 6 volumes of 95% ethanol, extracted with 0.1 N acetate buffer (pH 4.8), and precipitated differentially with acetone, the fraction precipitating between 40-70% being saved. This precipitated fraction was dissolved in a small volume of 0.1 N acetate buffer. An extract of urine from young adult males was prepared by the procedure of Katzman(9).

To test possible cross reactions of the antiserum with gonadotropins of pituitary origin, an extract of human anterior pituitary tissue stored in acetone at 4°C was prepared by a modification of the method of Woods(10). The tissue was extracted with acetone, dried and then extracted with 40% ethanol.

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|| The commercial human chorionic gonadotropin (Follutein) employed was supplied in generous quantities by Dr. Aleck Borman, Suibb Inst. for Medical Research, New Brunswick, N. J.

The ethanol extract was dialyzed against 0.1 N acetate buffer (pH 4.8), and the gonadotropin-rich fraction precipitated with 70% acetone and solubilized by dialysis against 0.01 M phosphate buffered 0.15 M NaCl (pH 7.0). The extracts were designated as follows: male urine extract (MUE), anterior pituitary extract (APE), concentrated pregnant human serum (C-PHS), concentrated normal human serum (C-NHS), and concentrated sera from hamsters bearing human tumors, Pitt 89 (C-89), Pitt 100 (C-100), and Deac 3 (C-D3).

All sera and extracts were stored at either 4°C or –20°C after addition of merthiolate (1:10,000).

Double diffusion and immunoelectrophoresis in agar. Double diffusion in agar was accomplished by a modification of the Ouchterlony technic(11), using 0.7% Ionagar No. 2¶ in veronal buffered 0.15 M NaCl (pH 8.2) containing 1.0 M glycine and 1:10,000 merthiolate.

Immunoelectrophoresis was performed according to a modification of the method of Grabar and Williams(12), employing 1.25% Ionagar No. 2 in veronal buffer at pH 8.2 with an ionic strength of 0.04. The reactants were mixed with an equal volume of 2.5% Ionagar No. 2 and placed in wells. Following electrophoresis (1 hour at 18 volts/cm) troughs were cut and filled with undiluted antisera. For development of precipitin bands, the immunoelectrophoretic plates and the double diffusion plates were incubated at 4°C. They were photographed and finally dehydrated and stained with azocarmine G or ponceau S for a permanent record.

To remove antibodies to impurities detected by the immunodiffusion technics, antisera were absorbed by adding 0.05 ml of NHS or MUE to each ml of antiserum. This mixture was incubated at 37°C for 30 minutes, followed by incubation at 4°C overnight. Resulting precipitates were removed by centrifugation. This procedure was repeated until no precipitate could be detected and no precipitin bands could be seen against the NHS or MUE on double diffusion in agar.

Bioassay and neutralization studies. The biological activity of HCG in various preparations was determined in weanling male rats by measuring changes in mean prostatic weight. The preparations were injected subcutaneously daily for 4 days and the wet weight of the ventral lobes of the prostates determined 24 hours after the last injection. Results were expressed as milligrams of prostate per 100 g of body weight. The commercial HCG used to prepare the antiserum served as the standard. Using this method PHS assayed 64 I.U./ml; C-PHS, 500 I.U./ml; and PHU, 8 I.U./ml. APE contained gonadotropic activity equivalent to 730 I.U. of HCG/ml.

Neutralization studies were performed by mixing 1 ml dilutions of antiserum in normal rabbit serum with 1 ml of either PHU or PHS, the latter preparations diluted in saline to contain approximately 4 I.U. of HCG/ml. The preparations were incubated for one-half hour at 37°C followed by incubation overnight at 4°C. Each rat received 4 I.U. of HCG plus varying amounts of antiserum in a total volume of 2.0 ml over a period of 4 days (0.5 ml × 4 days).

Results. Agar-gel double diffusion studies of pooled rabbit anti HCG demonstrated that the commercial HCG employed for immunization contained several contaminating antigens. From 2 to 5 precipitin bands were formed upon interaction of anti HCG with: MUE, C-NHS, NHS, C-PHS, PHS, C-89, C-100 and HCG-U. No precipitin bands could be detected with APE, with concentrated sera from hamsters heterotransplanted with a non-gonadotropin secreting human embryonal carcinoma (C-D3), or with sera from mice in early or late gestation. A common band of identity was detected against all preparations containing HCG (PHS, C-PHS, C-100, C-89 and HCG-U).

This common band of identity was shown with gel diffusion studies employing antiserum absorbed with either NHS or MUE to be the result of the reaction of HCG with its specific antibody. Antiserum absorbed with NHS produced a single band of identity with all sera or serum concentrates containing HCG, while no bands could be detected against NHS, C-NHS, APE or C-D3 (Fig.

¶ Consolidated Laboratories, Inc., Chicago Heights, Ill.

TABLE I. Neutralization of Biological Activity of HCG in PHU and PHS As Determined by the Effect upon Prostate of Rat.*

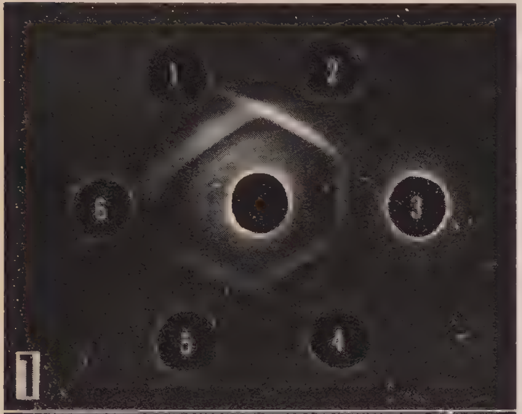
Gonadotropin source	Antiserum	Control NRS†	Dilutions of antiserum in NRS 1:5	1:25	1:125	1:625	1:3125
PHU‡	anti HCG-U	107 ± 15	—	56 ± 4.8	60 ± 7.8	67 ± 9.4	118 ± 16
PHS§	”	113 ± 20	68 ± 9.7	66 ± 9.5	64 ± 5.7	66 ± 6.7	97 ± 14
PHU	anti HCG-U abs. with NHS	88 ± 11	—	50 ± 4.7	47 ± 6.8	59 ± 11	89 ± 14
PHU	anti HSA	104 ± 16	98 ± 17	116 ± 15	110 ± 12	—	—
PHS	”	104 ± 16	85 ± 12	99 ± 20	109 ± 11	—	—
PHU	anti HGG	102 ± 13	107 ± 15	96 ± 16	112 ± 18	—	—
PHS	”	102 ± 13	85 ± 16	100 ± 15	97 ± 14	—	—

* Figures in columns are mean weights of 8 prostates expressed as mg/100 g of body wt ± 1 stand. dev.

† NRS = Normal rabbit serum.

‡ PHU = Pregnant human urine diluted 1:2 with saline to give approximately 4 I.U. HCG/ml.

§ PHS = Pregnant human serum diluted 1:16 with saline to give approximately 4 I.U. HCG/ml.



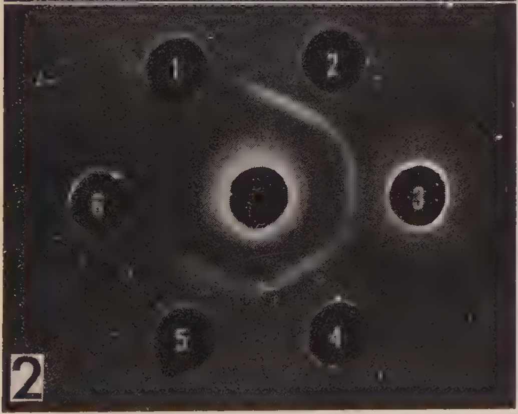


FIG. 1. Diffusion in agar of anti HCG absorbed with NHS (center well). Note common band of identity to all preparations containing HCG and non-related bands to MUE and HCG-U. Well 1 = MUE; Well 2 = HCG-U; Well 3 = PHS; Well 4 = C-100; Well 5 = C-89; Well 6 = NHS.

FIG. 2. Diffusion in agar of anti HCG absorbed with MUE (center well). A single band of identity appeared before all preparations containing HCG. Peripheral wells contain same reactants as in Fig. 1.

1). This common band of identity was also formed with HCG-U. Two or 3 additional bands, however, formed with MUE and HCG-U, indicating that anti HCG-U absorbed with NHS contained antibodies to contaminating antigens of urinary origin. Antiserum absorbed with MUE gave a single band of identity with all preparations containing HCG and no bands with MUE, NHS, C-NHS, C-D3 or APE (Fig. 2). The specific precipitin band appeared with concentrations of HCG as low as 16 I.U./ml. This level of hormone may be expected in serum and urine a week or so after the first missed menstrual period in early pregnancy.

Immunoelectrophoresis of HCG-U with non-absorbed anti HCG-U revealed 8 to 10 antigen-antibody precipitin bands. Similar results were obtained when another commercial preparation of HCG-U was employed (Pregnyl, Organon). After absorption of the antiserum with NHS, 3 bands were still detectable against HCG-U and 2 bands against MUE. This again indicated that HCG-U contained contaminating antigens common only to extracts of urine and not to serum.

When immunoelectrophoresis was performed on C-PHS and C-NHS with non-absorbed anti HCG-U, 2 closely associated bands migrating with alpha globulins were

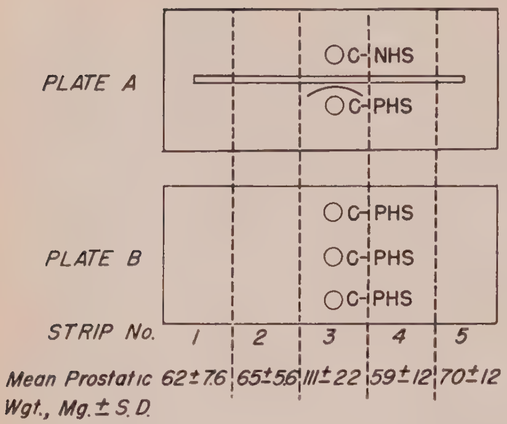


FIG. 3. Simultaneous (A) immunoelectrophoresis of C-PHS and C-NHS with anti HCG absorbed with NHS and (B) agar electrophoresis with subsequent bioassay of C-PHS to determine if the precipitin band to C-PHS migrated with the mobility of biologically active HCG. Saline extracts of each agar strip (Plate B) were bioassayed in 8 weanling rats and chorionic gonadotropic activity expressed as mean wt of ventral prostatic tissue in mg ± one stand. dev. Position of the single precipitin band to C-PHS (immunoelectrophoretic plate) corresponds to the agar strip containing maximal chorionic gonadotropic activity (electrophoresis plate).

formed against each preparation. In addition, a single band migrating with the beta globulins could be discerned against C-PHS. This electrophoretic mobility has been reported previously for HCG in the serum of pregnant women(13). When immunoelectrophoresis was repeated with anti HCG-U absorbed with NHS, only this latter band could be detected (Fig. 3, Plate A).

To determine if this single band migrated in the same manner as biologically active HCG, an agar plate containing C-PHS in 3 wells was subjected to simple electrophoresis in agar. Following the electrophoresis strips of the agar were bioassayed for gonadotropic activity (Fig. 3, Plate B). Simultaneous immunoelectrophoresis using anti HCG absorbed with NHS was performed on a second plate containing C-NHS and C-PHS (Fig. 3, Plate A). Maximal chorionic gonadotropic activity was found in strip 3 of the simple electrophoretic plate which corresponded to the position of the single band in the immunoelectrophoretic plate (Fig. 3). This provided further evidence that the single band was the result of the interaction of biologically active HCG with its specific antibody. A t-test comparison of the difference in mean chorionic gonadotropic activity (prostatic weight) between strips 2 and 3 and between strips 3 and 4 each gave $p<0.001$.

The results of studies to determine the ability of anti HCG-U to neutralize the biological activity of HCG are listed in Table I. The biological activity of 4 I.U. of HCG in either pregnancy serum or urine was fully neutralized by 1 ml of a 1:625 dilution of absorbed or non-absorbed antiserum. To confirm this specificity further pooled rabbit anti-human serum albumin (anti HSA) containing 0.85 mg antibody nitrogen/ml and pooled rabbit anti-human serum gamma globulin (anti HGG) containing 0.93 mg antibody nitrogen/ml were tested for their ability to neutralize the biological activity of HCG. Concentrations as great as 1:5 gave no evidence of neutralizing ability against preparations of urine or serum of pregnancy.

Discussion. Immunization of rabbits with commercial HCG has resulted in production of an antiserum capable of identifying HCG and neutralizing its biological activity in serum of urine. These studies have demonstrated that commercial HCG-U contains contaminating antigens common to normal human urine and a few common to normal human serum. The presence of these non-related antigens presents a theoretic objection to employment of non-absorbed antiserum to commercial HCG-U in an immunologic test for pregnancy(14). Our studies clearly indicate, however, that an immunologic test for pregnancy is feasible, employing either an antiserum against a highly purified preparation of HCG-U as done by McKean(3) or, as in this study, a properly absorbed antiserum against commercial HCG-U.

The similarity in immunobiologic behavior between HCG from serum and from urine strongly suggests that the specific antibodies are reacting directly with the biologically active hormone and not with carrier proteins differing in serum and in urine as hypothesized by others(5). These observations also demonstrate an immunobiologic similarity between the gonadotropic secretion of benign and malignant trophoblast of placental and testicular origin.

Summary. This report describes the immunobiological characterization of an antiserum to commercial human chorionic gonadotropin. This antiserum identified human chorionic gonadotropin of benign or malignant trophoblastic origin in serum or urine and neutralized its biological activity.

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Effect of 2,4-dinitrophenol on Free Fatty Acid Uptake by Skeletal Muscle.* (26854)

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It was reported(1) that in the anesthetized dog during direct electrical stimulation the muscles of the hind leg showed a consistent uptake of free fatty acids (FFA) on the stimulated side. A metabolic change very similar to that induced by muscular contraction can be produced by dinitrophenol (DNP) poisoning. Although the detailed mechanism of action of this "uncoupler" on the electron transport system is not yet entirely understood and the question whether it inhibits the formation of energy rich P bonds or enhances the hydrolytic cleavage of the terminal phosphate of ATP is a matter of discussion(2,3,4), it can be safely stated that DNP induces an imbalance between formation and breakdown of the high energy P compounds of the cell. The result of this imbalance is a dramatic rise of the metabolic rate.

Experiments carried out with a method similar to that used in this study had shown that in the anesthetized dog DNP increased blood flow, oxygen uptake and lactic acid production of the muscle 5-9-fold glucose uptake was increased about 2-fold, and the muscle glycogen decreased about 40% in 60 minutes. A considerable loss of inorganic phosphate and a concomitant rise of plasma inorganic P level were also recorded(5,6).

The muscle creatine phosphate content dropped 59% while ATP content decreased only 13%(5,6). As far as the metabolism of muscle is concerned, DNP poisoning can be considered as exhausting work lasting for about 1-2 hours, the only difference being that the liberated energy is wasted in the form of heat without performing work.

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* Supported by Nat. Heart Inst.

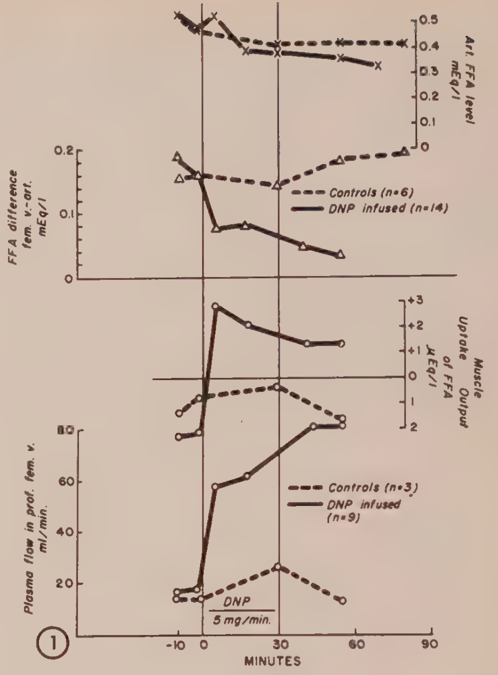
Earlier investigations(7) suggested that DNP may increase oxidation of fat in the unanesthetized intact animal. DNP decreased the RQ of rats from 0.93 to 0.78. It was also shown in dogs that the increase in O_2 uptake induced by DNP was considerably higher than would have been required for oxidation of carbohydrate which disappeared from the body. Since N excretion was not increased, the conclusion was drawn that DNP accelerated oxidation of fat. Considering these findings it was of interest to study the uptake of FFA by the muscle under the effect of DNP.

Methods. Adult mongrel dogs (body weight: 15-25 kg) of both sexes were studied in the post-absorptive state under sodium pentobarbital anesthesia (30 mg/kg i.v.). Blood flow was determined according to the method of Issekutz Jr. *et al.*(8) in the right profunda femoris veins which drain predominantly muscular areas. Plasma flow was calculated from simultaneous determinations of blood flow and hematocrit.

In 9 animals estimated hepatic plasma flow (EHPF) was determined by the BSP dilution method of Bradley *et al.*(9) measuring portal and hepatic venous blood BSP levels (10).

Blood samples were taken simultaneously from the following vessels: right femoral artery, and right profunda femoris, left femoral, portal and hepatic veins. The blood samples were placed in chilled oxalated tubes, centrifuged, separated, and precipitated in the cold. FFA was determined in duplicate analyses by the method of Dole(11).

DNP solutions for infusion were prepared by dissolving the commercial preparation in water made alkaline by addition of a few drops of 1 N NaOH. In control experiments infusion of a salt solution with pH adjusted to that of the DNP mixture was used. Both DNP and BSP infusions were given with Harvard constant rate infusion pumps. The dose of DNP was adjusted so that each animal received 2 ml/min of a solution containing 2.5 mg/ml for 30 minutes. Intra-arterial infusions were made into the femoral artery just distal to the profunda femoris branch, so that the drug would be carried directly to the area supplied by the latter vessel.



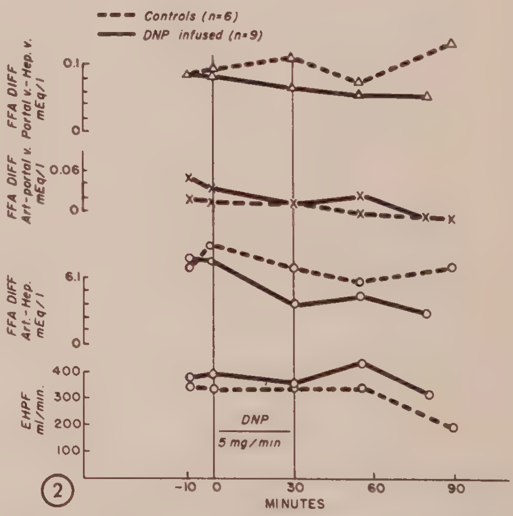


FIG. 1. Effect of DNP on plasma flow and FFA uptake in profunda femoris veins.

FIG. 2. Effect of DNP on hepatic uptake of FFA.

Results. Fig. 1 shows the effect of DNP on plasma flow in the profunda femoris vein, FFA output and uptake by this area, arteriovenous (femoral vein) FFA difference in the other leg and arterial FFA level. The curves represent average values obtained in 9 animals. Plasma flow was increased by intra-arterial infusion of DNP to 4-5 times the initial value. At the same time the FFA output of the area which was found in all dogs prior to infusion, was changed to a definite FFA uptake in 8 of 9 experiments. The mean difference between pre-infusion FFA output and average uptake measured in the first 60 minutes after the start of DNP infusion was 3.83 μEq/min with a standard error of ±0.9 μEq/min. This difference was significant at the $p<0.01$ level.

Blood of the femoral vein always contained more FFA than arterial blood. This difference decreased after DNP administration to about one-fifth the original value. The mean difference and its standard error was 0.128 ±0.026 mEq/l. This change is significant at the $p<0.001$ level.

The slow decrease of arterial level from 0.48 mEq/l to 0.33 mEq/l shown by the average curve is not significant. Of 14 dogs it was decreased in 6, increased in 5 and unchanged in 3. In 9 experiments hepatic plasma flow was also estimated (EHPF) and FFA differences (mEq/l were measured across the splanchnic area (artery-portal vein) and across the hepatic portal system (Fig. 2). No striking change was observed during DNP poisoning. The decrease of arterio-hepatic FFA difference shown on the average curve is not significant (average decrease: 0.061 ± 0.031 mEq/l; $0.05<p<.1$). Since the possibility could not be ruled out that DNP poisoning limited the validity of the indirect estimation of hepatic plasma flow, a calculation of hepatic FFA uptake does not seem warranted.

Discussion. Blood in the profunda femoris veins carries more FFA than arterial blood, meaning that FFA uptake by the skeletal muscle of the anesthetized dog at rest is either zero or so low that the continuous release of FFA from the adipose tissue present in this area can mask it entirely. However, when the energy requirement of the muscle increases and turnover rate of the tricarboxylic acid cycle in turn increases many fold (either due to electrical stimulation(1) or to administration of DNP), then FFA uptake by muscle becomes measurable and the blood in the profunda femoris veins contains less FFA than the arterial blood.

After administration of DNP the femoral vein—arterial FFA difference decreases strikingly. Concentration in the former, however, remains higher than in the arterial blood. Two factors may contribute to this effect: (a) increased blood flow and (b) increased FFA uptake of the muscles drained by the femoral vein.

The alternative explanation that FFA output by the adipose tissue of the leg was decreased seems unlikely because (a) hepatic uptake of FFA did not seem to show a significant decrease; (b) FFA uptake by the muscle was elevated; and (c) considering the small pool size of the albumin bound FFA, a decreased release would be expected to induce a sharp drop of arterial FFA level. This was not the case.

Summary. Uptake of free fatty acids by skeletal muscle was determined by simultaneously measuring blood flow through profunda femoris veins, and arteriovenous fatty acid differences in anesthetized dogs. Administration of 2.5 mg/ml of DNP for 30 minutes caused elevation of blood flow and uptake of free fatty acids by the skeletal muscle.

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Study of Emetic Receptor Sites for Staphylococcal Enterotoxin in Monkeys.** (26855)

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A medullary emetic center (MEC) which lies in the tractus solitarius and its nucleus and the dorsolateral border of the lateral reticular formation is needed for vomiting with all emetics. This center is neither motor nor sensory, but coordinates the activities which constitute the emetic act. Each type of emetic has its own primary locus of action. The early vomiting following oral administration of copper sulfate results from gastrointestinal irritation(1). The centrally acting emetics such as apomorphine and Hydergine (equimixture of 3 dihydrogenated ergot alkaloids) initiate vomiting by acting at the chemoreceptor trigger zone (CTZ) which lies in, or is immediately adjacent to, the area postrema on the floor of the fourth ventricle (2).

Only cats have been used in the published reports on the site of emetic action of staphylococcal enterotoxin. Acute bilateral vagotomy greatly reduced the incidence of vomiting in cats injected intraperitoneally with crude enterotoxic filtrates, indicating peripheral sensory receptors in the viscera which transmit through the vagi(3). A review of the data resulted in an emphasis on a possible forebrain receptor site(1). Chronic ablation of the CTZ did not prevent emesis following intravenous injection of a highly purified staphylococcal enterotoxin, but chronic midbrain transection resulted in refractory animals(4).

Monkeys may differ from cats as to the receptor sites for emesis. Rhesus and cynomologus monkeys do not vomit in response to the emetics known to act on the CTZ of dogs and cats(5). Nevertheless, either vagotomy or CTZ ablation protects monkeys against the early emesis following a single total body X-irradiation of 1200 r(6), while CTZ destruction in cats does not confer protection against radiation emesis(7).

Two other observations suggested the present study on the vomiting response to staphylococcal enterotoxin of monkeys subjected to area postrema ablation or vagotomy. A higher incidence of vomiting occurs in monkeys being challenged with enterotoxin when the animals are administered a non-emetic dose of dihydroergotamine methanesulfonate (8), a drug closely related to Hydergine, which is a CTZ emetic in dogs(2). Perphenazine is a potent anti-emetic in dogs against apomorphine and Hydergine induced vomiting; the antiemesis may be due to the competition with the emetics for the receptor sites of the CTZ(9). This drug effectively inhibits enterotoxin evoked emesis in monkeys in dosages not significantly depressing the medullary emetic center(10).

Materials and methods. The sensitivity of *Macaca mulatta* to the emetic stimuli of 2 antigenically different enterotoxins (partially purified and lyophilized enterotoxins prepared from the 196E and S-6 strains of staphylococci*) and copper sulfate was determined preoperatively by feedings of the emetics in 50 ml of H_2O at weekly intervals. Only those monkeys vomiting within 1 hr with 320 mg $CuSO_4 \cdot 5H_2O$ and within 3 hr with 100 μg S-6 and 750 μg 196E enterotoxins were used. When indicated by the latency, additional feedings with ½ the previous dosage of copper sulfate were carried out to determine more precisely the minimal emetic dose. No animal vomited to 80 mg of this salt. The postoperative vomiting response to the 2 types of emetics was determined after a convalescent period of 2-4 weeks.

Monkeys vary considerably in their origi-

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* Prepared by Dr. M. S. Bergdoll.

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nal susceptibility and develop resistance to enterotoxin. The post-operative challenging doses were estimated from previous experience in this laboratory on the increase of tolerance to enterotoxin and are approximations of the number of minimal emetic doses for a normal monkey. If an animal vomits 1 or 2 times at initial feeding of a given dosage of enterotoxin, it usually vomits when fed 2× the original dose at the next feeding. If it vomits 3-4 times, the minimal emetic dose at the second challenge is about the same as the first feeding; if 5 or more times, one-half of the initial dose will usually cause vomiting at the next feeding. A single feeding of a small dose of one serological type of enterotoxin does not significantly increase the resistance to the other enterotoxin, but a single large dose was assumed to increase the tolerance to the other enterotoxin 2-fold. The general validity of these assumptions in calculating the number of minimal emetic doses is indicated by monkeys which vomited in response to one enterotoxin but not to the other. In these instances emesis always occurred following challenge with the larger of the calculated doses.

CTZ ablation was accomplished by the method used on dogs(11), the bilateral lesions centering on the area postrema being made by suction. Bilateral vagotomy was performed under nembutal anesthesia. A midline incision was made from the xiphoid to the umbilicus and the esophageal hiatus widened. The esophagus was mobilized and the main trunks of the vagus transected as high as possible. The serosa around the esophagus was stripped and cut circularly to interrupt nerve fibers that may have been intact. Pyloroplasty by the method of Heineke-Mikulicz(12) was done to facilitate the emptying by the vagotomized stomach.

The animals were sacrificed after completion of the post-operative feeding tests. The vagotomized animals were dissected to ascertain grossly the completeness of vagotomy. The lesions in the brain stem were localized by a study of serial histological sections stained by Masson trichrome method.

Results. Monkeys with the area postrema destroyed are highly tolerant of the emetic stimulus of staphylococcus enterotoxin, this resistance being effective against both serological types of the toxin (Table I).

TABLE I. Vomiting Response to Orally Administered Staphylococcal Enterotoxin of Area Postrema Ablated Rhesus Monkeys.

Monkey No.	Enterotoxin dosage and response*
498	Resistant to 20 MED 196E and 20 MED S-6
550	" " 20 " " " 30 " S-6
628	" " 35 " " but not to 60 MED S-6
627	" " 25 " " and 50 MED S-6
787	" " 70 " " " 100 " "
791	" " 90 " " " 120 " "
795	" " 100 " " " 100 " "

* Challenging dose in numbers of minimal emetic doses (MED) if animal had not been surgically treated. Estimated from preoperative responses and corrected for avg increase in resistance resulting from previous enterotoxin feedings.

Area postrema ablation increased the tolerance of $CuSO_4$ to some degree; 2-3-fold greater dosage than preoperatively was usually required to obtain vomiting within the same latency. These results indicate that the MEC is still functional. In none of the animals was there histological evidence that the experimental lesion extended into the region of the vomiting center.

Some interference with the dorsal sensory nucleus of the vagus was found in some of the animals. However, there was no correlation between this extension of the lesion and resistance to staphylococcal enterotoxin. Fig. 1 shows lesions produced in 4 of the animals listed in Table I.

Monkey 628 is especially informative. There was negligible damage to the sensory nucleus of the vagus, but the outline of the area postrema on one side was intact. The resistance of this animal to 35 minimal doses, but not to 60, correlates well with the altered histology of the remaining area postrema. The area postrema must, therefore, be an important site for enterotoxin induced emesis.

Monkeys subjected to vagotomy showed increased tolerance of the emetic stimulus of both serological types of staphylococcal enterotoxin (Table II). Complete protection was not obtained in all animals, but other animals did not vomit in response to the feed-

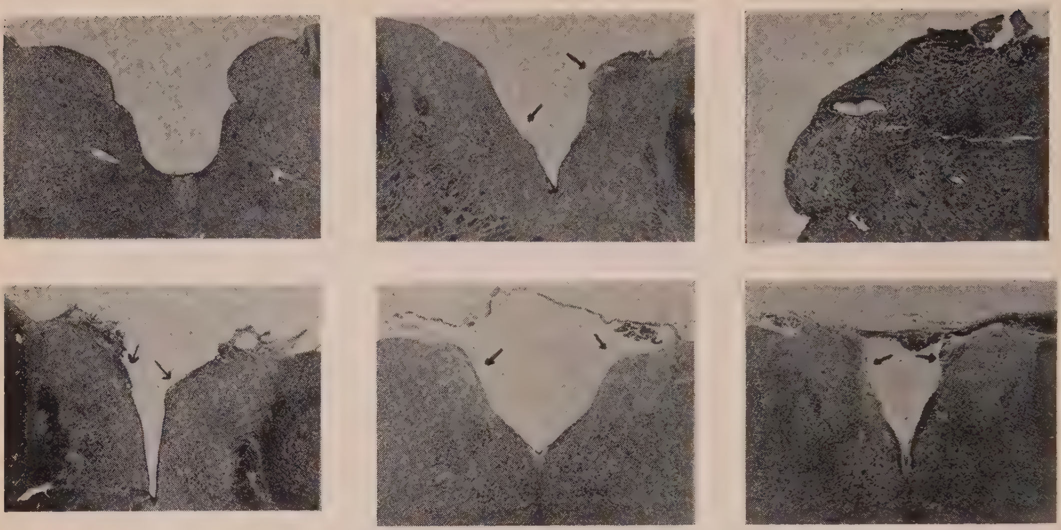

FIG. 1. Sections of medulla at level of area postrema. Left to right, top row: normal animal, monkey # 628, and enlargement of area postrema on right side of #628, showing extensive deterioration of normal structure. Bottom row: monkey #627, 787, and 795. See text for feeding results.

ing of up to 100 minimal emetic doses. The resistance to orally administered copper sulfate was raised so that an increase in dosage to about 2 times the preoperative level was required to obtain emesis or the latency with the equivalent dose was 2 or 3 times longer.

Discussion. Surgical destruction of the area postrema of rhesus monkeys resulted in complete refractoriness of the monkeys to feeding of staphylococcal enterotoxin. This induced resistance was effective against both antigenic types of enterotoxin used. The responsiveness of these monkeys to orally administered copper sulfate and the study of the brain section showed that the vomiting center and motor components of the vomiting reflex were functional.

Vagotomy at the level of the diaphragm gave complete resistance in some monkeys but not in others. This may be due to incomplete vagotomy, although no macroscopically intact fiber was evident. The partial protection in monkeys 629 and 634 and the protection against the very high challenging doses in the last 3 animals of Table II would favor this explanation. These results with enterotoxin would then parallel those obtained in the refractoriness of monkeys to X-irradiation induced emesis following either vagotomy or CTZ ablation(6).

The proximity of the dorsal sensory nucleus of the vagus to the area postrema and the similarity of the protection achieved by area postrema destruction or vagotomy make difficult the proof for existence of 2 emetic receptor sites for enterotoxin in the monkey. The histological evidence indicates that CTZ ablation without significant encroachment of the lesion into the dorsal sensory nucleus confers effective protection against enterotoxin induced vomiting. X-irradiation-induced emesis was not eliminated in a monkey with severe damage to the dorsal sensory nucleus without appreciable damage to the area postrema(6). However, vagal element or pre-

TABLE II. Vomiting Response to Orally Administered Staphylococcal Enterotoxin of Chronically Vagotomized Monkeys.

Monkey No.	Enterotoxin dosage and response*
577	Resistant to 20 MED 196E and 20 MED S-6
629	" " 15 " " but not to 40 MED S-6
630	" " 20 " S-6 but not to 30 MED 196E
634	" " 25 " 196E and 40 MED S-6
794	" " 50 " " " 100 " "
796	" " 80 " " " 100 " "
865	" " 70 " " " 70 " "

* See footnote of Table I.

reticular structures necessary for vomiting following X-irradiation or oral administration of enterotoxin may traverse the area postrema of the monkey. On the other hand, if complete protection results from complete vagotomy at the diaphragm level, then the termination near the ala cinerea of the thoraco-, lumbo-, and sacro-bulbar tracts(13) would not be important in enterotoxin induced vomiting in rhesus monkeys.

However, the incomplete protection in some of the vagotomized monkeys may be significant. One of 8 cats with bilateral vagotomy performed supradiaphragmatically at a level where the recurrent laryngeal nerves are also cut still vomited after intraperitoneal injection of enterotoxic filtrate(2). If the incomplete protection of monkeys is not due to partial vagotomy, the importance of the integrity of the CTZ for the vomiting stimulated by enterotoxin is enhanced. In either case, monkeys would differ from cats, since CTZ-destroyed cats still vomited to intravenous enterotoxin(4). Whether the parenteral injections of enterotoxin into cats as compared to the intragastric route used in the monkeys accounts for the difference is unknown. The ineffectiveness of CTZ ablation in cats as contrasted to the protection in dogs against the emesis resulting from X-irradiation(7) and nitrogen mustard(14) further suggests species differences. A forebrain site for emesis due to enterotoxin as demonstrated in cats(4) would seem to be lacking in monkeys, unless it serves a facilitatory function.

Summary. Bilateral destruction of the area postrema on the floor of the fourth ventricle (chemoreceptor trigger zone) makes rhesus monkeys completely refractory to the emetic action of staphylococcal enterotoxin. The surgically induced resistance is effective against the 2 antigenic types of enterotoxin used. Vagotomy at the level of the diaphragm gave protection in all animals, but in some protection was not complete. The partial protection against enterotoxin-induced emesis in some of the vagotomized monkeys may be due to incompleteness of the vagotomy. If this interpretation of the results is correct, a comomn basis of emesis prevention following staphylococcal enterotoxin feedings may account for the similarity of vomiting response of the area postrema ablated and vagotomized animals. A species difference between cats and rhesus monkeys in the importance of the area postrema region for enterotoxin stimulated vomiting is indicated.

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Inhibition of Hepatic Alanine Transaminase Activity in Response to Treatment with 11-Deoxycorticosterone.* (26856)

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Alanine transaminase activity is increased markedly in the liver of rats treated with glucocorticoids or corticotropin(1-3). In contrast, treatment with deoxycorticosterone (2) or adrenalectomy(4) caused a significant reduction in level of this transaminase in liver. The stimulatory action of cortisol on hepatic alanine transaminase activity was not impaired when this steroid was given in combination with deoxycorticosterone(2). The purpose of this study was to determine whether the fall in liver alanine transaminase following administration of deoxycorticosterone is related to its inhibitory effect on release of corticotropin by the pituitary.

Materials and methods. Male hypophysectomized rats of the Sprague Dawley strain, obtained from the Charles River Laboratories were used. Rats were adrenalectomized 5 days prior to use and were given either 11-deoxycorticosterone or one per cent saline in place of drinking water. Deoxycorticosterone was administered subcutaneously as an aqueous suspension. Corticotropin (10 I.U. per injection, 20 I.U., total dose) (ACTHAR GEL, Armour Pharmaceutical Co.) was administered twice daily. Purina Laboratory Diet was fed *ad libitum* to all animals.

The animals were stunned by a blow on the head, then killed by exsanguination. The livers were immediately placed in beakers on crushed ice. Homogenates were prepared in a Waring Blendor with ice-cold distilled water. Alanine- and aspartate-α-ketoglutarate transaminase activities were measured by procedures(2) similar to those described by Wroblewski and La Due(5) except that instead of measuring the oxidation of DPNH spectrophotometrically, DPN was determined by a fluorometric procedure developed by Lowry and co-workers(6). Tyrosine-α-ketoglutarate transaminase activity was determined using a modification of the method described by Sereni and co-workers(7). Protein was determined by a modification of the Folin procedure(8).

Results. The data presented in Table I indicate that treatment of rats with deoxycorticosterone (DOC) causes a marked reduction in liver alanine transaminase activity. Administration of DOC to adult rats depressed the activity of this transaminase to 42% of control value. Treatment of younger animals with DOC also lowered the values of this transaminase in liver to 30% of normal.

The depression in hepatic alanine transaminase activity which followed adrenalectomy was comparable to that observed in unoperated rats treated with deoxycorticosterone (Table I). In contrast to the effect of DOC on alanine transaminase activity in intact animals, treatment of adrenalectomized rats with DOC for 14 days did not further reduce the activity of this enzyme in liver.

On the basis of these results, a direct effect of DOC on alanine transaminase appeared unlikely. The possibility that DOC might act by impairing the secretion of corticotropin by the hypophysis was tested by administration of DOC to hypophysectomized rats. Hepatic alanine transaminase values were 2 to 5 times higher in hypophysectomized rats than in intact rats of similar age and weight, whereas the values for tyrosine and aspartate transaminase in liver were not significantly altered by hypophysectomy (Table II). Treatment of hypophysectomized rats with DOC did not reduce the activity of alanine transaminase in liver nor did it affect the activity of either tyrosine or aspartate transaminase.

When hypophysectomized animals were treated with corticotropin, the activity of alanine and tyrosine transaminase in liver was

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TABLE I. Effects of Deoxycorticosterone and Adrenalectomy on Hepatic Alanine Transaminase Activity.

	Body wt (g)	Liver wt (g)	Alanine transaminase (mMoles of substrate utilized/hr)	
			Per g wet wt	Per g protein
Intact, no treatment	407	14.14 ± .71*	2.79 ± .50	17.0 ± 2.8
" , 3 mg DOC daily for 7 days	374	11.03 ± .75	1.26 ± .42	7.2 ± 2.4
Intact, no treatment	195	8.82 ± 1.12	1.82 ± .41	10.7 ± 2.0
" , 3 mg DOC daily for 14 days	201	9.08 ± 1.22	.58 ± .23	3.27 ± 1.1
Adrenalectomized, no treatment	132	5.29 ± 1.23	.52 ± .23	3.20 ± .91
" , 3 mg DOC daily for 14 days	170	7.37 ± 1.06	.45 ± .26	2.44 ± 1.1

* Avg value of 5 or more rats/group ± stand. dev.

increased several-fold (Table II). Aspartate transaminase activity was not changed by administration of corticotropin. In hypophysectomized animals injected with both deoxycorticosterone and corticotropin, 2-fold increases in hepatic alanine and tyrosine transaminase activity were observed (Table II). An effect of DOC in suppressing the response to ACTH was questionable. In a similar experiment, however, corticotropin administered to hypophysectomized rats increased liver alanine transaminase activity to a value of 57.9 ± 7.0 mMoles of substrate per g protein per hour while treatment with both deoxycorticosterone and corticotropin gave a value of 52.4 ± 5.3. Thus, the response to treatment with both hormones was comparable to that obtained when corticotropin only was administered.

To insure that the effect of corticotropin on liver alanine transaminase activity was mediated through the adrenal gland, this hormone was administered to adrenalectomized rats. Treatment of adrenalectomized rats with 20 units of corticotropin daily for 7 days did not significantly alter liver alanine transaminase activity (Table II).

Discussion. Deoxycorticosterone appears to affect the activity of alanine transaminase of liver indirectly by interfering with release of corticotropin from the hypophysis. In support of this hypothesis is the failure of deoxycorticosterone to alter hepatic levels of alanine transaminase in adrenalectomized rats, in contrast to the marked decrease in the activity of this transaminase produced in unoperated animals by the same treatment. Previous studies indicated that deoxycorticosterone did not interfere with the response of alanine transaminase to cortisol(2), nor could the effect of deoxycorticosterone be attributed to loss or binding of pyridoxal phos-

TABLE II. Effect of Deoxycorticosterone and Corticotropin on Hepatic Transaminase Activity of Hypophysectomized Rats.

Treatment		Body wt (g)	Liver wt (g)	Transaminase activity (mMoles substrate utilized/g protein/hr)		
				Alanine	Tyrosine	Aspartate
Intact		99	3.64 ± .26†	4.30 ± .97	.60 ± .15	127 ± 6
Hypophysectomized		102	3.78 ± .29	23.4 ± 1.2	.77 ± .10	95 ± 5
"	+ DOC*	93	3.97 ± .28	21.9 ± 2.5	.65 ± .26	114 ± 15
"	+ ACTH*	99	4.09 ± .30	58.3 ± 8.6	2.32 ± .47	149 ± 21
"	+ DOC + ACTH*	87	4.23 ± .69	40.8 ± 9.9	1.43 ± .64	103 ± 30
Adrenalectomized		150	4.58 ± .68	5.13 ± 1.4	—	—
"	+ ACTH*	174	6.36 ± .63	3.50 ± .45	—	—

* DOC = Deoxycorticosterone, 3 mg administered subcut. daily for 7 days. ACTH = Corticotropin, 10 units administered subcut. twice daily for 7 days.

† Avg value of 5 rats/group ± stand. dev.

phate, the coenzyme of alanine transaminase.†

In previous studies in this laboratory corticotropin increased hepatic alanine transaminase activity of intact rats(3). The effect of corticotropin on this transaminase is most likely mediated through the adrenal gland since no alteration in enzyme activity was noted when corticotropin was administered to adrenalectomized animals (Table II). The slight reduction in alanine transaminase response observed when deoxycorticosterone was administered simultaneously with corticotropin was not statistically significant. Recently it has been reported that corticosterone may inhibit adrenal steroid production in the rat by acting directly on the adrenal gland(9). The results of the present work do not exclude the possibility that deoxycorticosterone may act in a similar manner.

The release of corticotropin by the pituitary following cold stress or unilateral adrenalectomy has been found to be inhibited by treatment with corticosteroids(10-12). Sayers and Sayers(10) and Long(13) observed that the depletion of adrenal ascorbic acid following stress could be prevented by previous treatment of animals with adrenal steroids. Recently several assays for corticotropin have been based on the inhibition of its release from the pituitary by treatment with a glucocorticoid(14,15). Deoxycorticosterone also blocks the secretion of corticotropin by the pituitary(11,12) but has only one-eighth the potency of cortisone in this respect(10). The results obtained with deoxycorticosterone have been variable(16-18). Route of administration of deoxycorticosterone or severity of the stress stimulus(19) appear to be important factors concerning the relative suppression of corticotropin output.

The finding that deoxycorticosterone lowered the activity of alanine transaminase in liver was of interest since other cortisol responsive tissues(20) or systems(21) are either refractory or show a weak glucocorticoid response after treatment with this mineral corticoid.

There are numerous reports concerned with the effect of corticosteroids on enzyme activity, among them the effects of cortisone and cortisol on enhancement of activity of several transaminases, such as tyrosine-(22, 23), tryptophan-(24,25), and alanine-α-ketoglutarate transaminase(1-3,26) and of tryptophan pyrrolase(27,28). Subsequent studies demonstrated that the high endogenous levels of activity of alanine transaminase in the liver of adult rats were dependent on presence of the adrenal gland(4). Deoxycorticosterone was found to depress the basal level of alanine transaminase in liver(2) but to be without effect on tryptophan pyrrolase(29).

While the activity of tryptophan pyrrolase has been reported to be decreased following adrenalectomy(28), the level of tyrosine transaminase in liver was not altered after adrenal ablation(7).

The changes in alanine transaminase activity induced by treatment with deoxycorticosterone or cortisol provide a new technic for studying the inter-relationships of these corticosteroids with other hormones and systems *in vivo*. Changes in activity of this transaminase may be correlated with the physiological effects of cortisol and deoxycorticosterone. For example, it has been noted that the growth inhibition of Walker carcinoma 256 in rats treated with cortisol is associated with a 10-fold rise in tumor alanine transaminase activity whereas slight stimulation of tumor growth and significant depression in transaminase activity occurred following administration of deoxycorticosterone(30). In the present study, measurement of hepatic alanine transaminase activity after treatment with deoxycorticosterone was used as the basis for demonstrating that this hormone suppresses corticotropin secretion by the pituitary.

Summary. Daily administration of DOC for short periods resulted in a 60 to 70% depression in liver alanine transaminase activity. Adrenalectomy also caused a decrease in activity of alanine transaminase, but no further reduction in the level of this enzyme was observed upon treatment with DOC. Treatment with ACTH resulted in an increase in alanine transaminase activity in hy-

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† F. Rosen and R. J. Milholland, unpublished data.

pophysectomized rats, but not in adrenalectomized animals. Administration of DOC to hypophysectomized rats failed to lower alanine transaminase, nor did it alter the response of this enzyme to treatment with ACTH. The inhibitory effect of DOC on alanine transaminase activity appears to be due to suppression of ACTH release by the pituitary.

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Diminished Adrenal Corticoid Response to Burn and ACTH in the Nephrectomized Dog.* (26857)

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Recent reports have implicated the kidney in the control of aldosterone secretion(1,2,3), and have suggested that this organ may play some role in control of 17 hydroxycorticoid (17 OHCS) secretion as well(3,4). Since ACTH is known to affect both aldosterone and 17 OHCS secretion, the present studies were undertaken to determine whether nephrectomy alters the 17 OHCS response to burn, a known stimulus to ACTH release, and if so, by what means.

Methods. Experiments were performed on

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22 dogs anesthetized with sodium pentobarbital. The right lumboadrenal vein was cannulated by the method of Hume and Nelson(5). Simultaneous bilateral nephrectomy was carried out in 10 dogs. One liter of normal saline was given intravenously through a femoral venous cannula to prevent hypovolemia during preparation. Arterial pressure was monitored by means of a cannula in the femoral artery. Following completion of the preparation, the animal was heparinized and left undisturbed for one hour. The level of anesthesia was maintained. Blood lost through adrenal venous collections or incidentally was replaced with dextran in saline.

Burn experiments were carried out in 6 normal and 7 nephrectomized dogs. Of these, 4 normal and 3 nephrectomized dogs were burned twice; all other dogs were burned once. In all cases of 2 burns, the magnitude of response to the second burn was equal to that from the first. A control timed sample of adrenal venous blood was first collected. The animal was then burned on the thigh with cautery for 2 minutes. This procedure resulted in a uniform third degree burn measuring more than 35 sq. cm. The edges of the burned area were sharp and there appeared to be virtually no region of submaximal burn. Additional timed adrenal venous samples were collected 10, 30 and 60 minutes after the start of the burn. In those dogs which were burned twice, the second burn (contralateral thigh) was begun after collection of the 60-minute post-burn sample. The same protocol was then observed following the second burn.

Nine ACTH experiments were carried out in 5 normal and 4 nephrectomized dogs. A control sample of adrenal venous blood was collected and 10 mμ of ACTH (Armour, crystalline, dissolved in saline) was injected into the adrenal vein over a 15-second period and washed in with 10 ml of saline. A second sample of adrenal venous blood was collected beginning exactly 5 minutes after ACTH injection. This collection extended over the period of maximal response described by Nelson and Hume(6). After a recovery period of 30 to 45 minutes, the procedure was repeated with a dose of 100 mμ; then, after a similar period, with a dose of 1 μ; then, after another period, with a dose of 22 μ. Hypophysectomized dogs were not used for these experiments because the intent was to determine the effect of the absence of kidneys alone. A series of increasing doses was used in the same animal in order to demonstrate the cumulative, as well as the acute, effects of ACTH.

Adrenal venous samples were centrifuged immediately, the plasma was withdrawn and analyzed for 17 OHCS by Peterson's modification(7) of the method of Silber and Porter (8). Secretion rates of 17 OHCS were calculated from concentration, rate of adrenal blood flow and hematocrit.

Results. Average initial control levels of 17 OHCS secretion were 2.67 ± 0.61 (SEM) μg/min for all normal dogs and 3.99 ± 0.61 μg/min in all nephrectomized dogs. These values are not significantly different. Adrenal blood flows were equal in the 2 groups.

The effects of burns upon adrenal 17 OHCS secretion in normal and nephrectomized dogs are shown in Fig. 1 and tabulated and analyzed in Table I. The difference between the 10-minute post-burn and control secretion rates is a measure of the response to burn, while the differences between the 30- and 60-minute post-burn and control secretion rates are measures of rate of return to control levels following stimulation. It may be seen that burn elicited a significantly greater rise in 17 OHCS secretion rates in normal than in nephrectomized dogs. Further, 17 OHCS secretion had returned to control levels after 30 minutes in nephrectomized dogs but were still elevated in normal dogs. 17 OHCS secretion rates 60 minutes after burn were not significantly different from control levels in either group. The high mean and large standard error at 60 minutes in the normal group are the results of a single experiment in which 17 OHCS secretion remained elevated. This was in an animal which was burned twice, and this result followed the second burn.

The effects of ACTH administration on adrenal 17 OHCS secretion rates are shown in Fig. 2 and tabulated and analyzed in Table II. It may be seen that ACTH led to a

TABLE I. a. Effect of Third Degree Burns on 17 OHCS Secretion (µg/min.) in Normal and Nephrectomized Dogs.

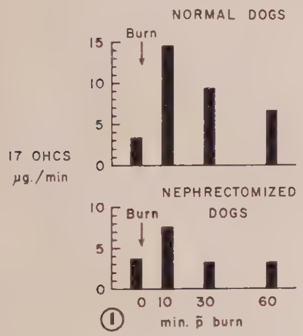
	No. of experiments	Control	10 min. after burn	30 min. after burn	60 min. after burn
Normal	10	3.29 ± .67*	14.47 ± 1.31	9.40 ± 2.20	6.68 ± 2.44
Nephrectomized	10	3.56 ± .55	7.52 ± .69	3.18 ± .74	3.15 ± .46

b. Analysis of differences in individual experiments

	Normal		Nephrectomized		Comparison, normal *vs* nephrectomized	
10 min. after burn minus control	11.18 ± 1.27	p <.001	3.96 ± .64	p <.001	t = 5.79	p <.001
30 min. after burn minus control	5.78 ± 1.87	p <.02	–.65 ± .54	p >.2	t = 3.31	p <.005
60 min. after burn	3.06 ± 2.43	p >.2	–.71 ± .50	p >.2	t = 1.51	p >.1

* Mean ± stand. error.

higher maximal secretion rate in normal than in nephrectomized dogs. The acute rise obtained with ACTH was, in addition, greater in normal dogs than in nephrectomized dogs in response to 10, 100 and 1000 mµ. This difference was obscured by the high control secretion rates in the group given 22 µ. The cumulative effect of multiple doses of ACTH, evidence by the rising control levels with repeated doses was also greater in normal than in nephrectomized dogs.



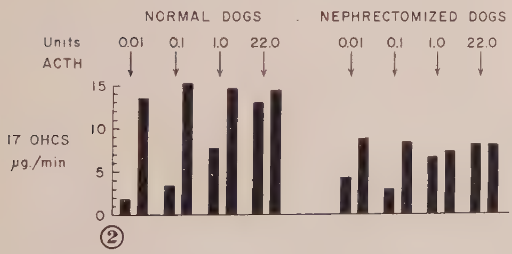


FIG. 1. Effect of burn on 17 OHCS secretion (µg/min.) in normal and nephrectomized dogs. Each bar is mean value (10 experiments in each group).

FIG. 2. Effect of ACTH in sequentially increasing doses on 17 OHCS secretion (µg/min.) in normal and nephrectomized dogs. Each bar is mean value (5 normal, 4 nephrectomized dogs).

Discussion. In general, dogs with chronic adrenal cannulae have been used for experiments such as those reported here. Though mean control secretion rates in the present experiments were slightly higher than those reported for dogs with chronic adrenal cannulae(5), there were many individual values below 2 µg/min, and the responses to stimuli were not prevented by the elevated control levels. Before starting this study, it was decided arbitrarily to exclude any dog with "resting" 17 OHCS secretion rate greater than 8 µg/min since a control level near to or at maximum secretion rate would obscure any response to a stimulus. Of 25 dogs prepared, only 3 were excluded because of this criterion. This proportion of animals lost does not seem to us excessive, compared to probable losses in chronic preparations from death, clotting, inadvertent removal of the cannula, and occasional very high control values, despite a 48-hour wait(5).

Third degree burns of the magnitude employed in these studies lead to rates of secretion of 17 OHCS equal to those achieved with large doses of ACTH in similar animals. The 17 OHCS response to burn is attenuated markedly in previously nephrectomized animals. This effect is not the result of different control secretion rates (Table I). It cannot be ascribed to physical damage to the adrenal in the course of nephrectomy, since adrenal blood flows were equal in the 2 groups, and

TABLE II. Effect of Sequentially Increasing Doses of ACTH on 17 OHCS Secretion (μg/min.) in Normal and Nephrectomized Dogs.

	Normal (5 dogs)			Nephrectomized (4 dogs)			Comparison, Δ normal *vs* Δ nephrectomized	
ACTH	Control	5 min. after ACTH	Δ*	Control	5 min. after ACTH	Δ	t	p
10 mμ	1.9	13.5	11.6 ± 1.8†	4.2	8.7	4.5 ± 1.3	3.8	<.01
100 mμ	3.4	15.2	11.8 ± 1.9	2.9	8.3	5.4 ± .5	3.3	<.02
1 μ	7.7	14.7	7.0 ± 1.6	6.6	7.3	.7 ± 1.0	3.2	<.02
22 μ	13.0	14.5	1.5 ± 1.1	8.2	8.1	–.1 ± .7	1.2	>.2

Mean secretion rate after ACTH: Normal, 14.47 ± .94; nephrectomized, 8.11 ± .48.
t = 6.01 p <.001

* Analysis of differences in individual experiments.

† Mean ± stand. error.

there was no impairment of resting secretion rates of 17 OHCS. This limitation of response could be explained either by decreased ACTH release by the pituitary or by decreased adrenal sensitivity to ACTH.

The ACTH experiments described were designed to distinguish between these possibilities. It is evident from these experiments that the maximum secretion rate in response to ACTH is reduced markedly in nephrectomized dogs. Both immediate response to injected ACTH and cumulative effect on 17 OHCS secretion rates of repeated doses of the drug are less in nephrectomized than in normal dogs. It seems reasonable to conclude that the adrenal glands of nephrectomized dogs are less sensitive to ACTH than are those of normal dogs, so far as 17 OHCS secretion is concerned. The different responses to burn of normal and nephrectomized dogs can be ascribed to diminished sensitivity to ACTH in the nephrectomized group. Indeed, the observed difference between the 2 burned groups (Table I) is almost quantitatively accounted for by the differences in maximal secretion rates (Table II), since burns led to apparently maximum secretion rates in both groups.

The diminished sensitivity to ACTH of adrenal 17 OHCS secretion in nephrectomized dogs may be explained in 2 principal ways, or in combinations of these. First, substances which inhibit adrenal corticoid secretion or synthesis might accumulate in the blood in the absence of the kidneys. The fact that the nephrectomized dogs reported here did not have lower control 17 OHCS secretion rates than did the normal dogs would seem to militate against this hypothesis, particularly since the control secretion rates observed in these studies were not absolutely basal, and are significantly different from zero (See, *e.g.* Table I).

Second, the kidney might secrete a substance which stimulates adrenal 17 OHCS secretion. Evidence suggests that the renin-angiotensin system plays such a role. Deane and Masson(9) reported that injection of renin into rats may cause hypertrophy of the zona fasciculata of the adrenal as well as of the zona glomerulosa. There are recent reports that both renin and angiotensin infused into hypophysectomized nephrectomized dogs may stimulate 17 OHCS secretion(2,3,10,11). ACTH may play a role in regulation of kidney renin content(11,12). Further, injected ACTH is rapidly bound to kidney tissue in large proportion(13). It is possible that part of its adrenotropic action is mediated through the renin-angiotensin system. However, it is also possible that angiotensin, released by mechanisms other than ACTH, may alter adrenal sensitivity to ACTH. It has been reported that renin is released in hemorrhagic shock(14,15). Hume and Nelson(5) studied 17 OHCS secretion during hemorrhagic shock in hypophysectomized dogs maintained on constant ACTH, and found an increase in 17 OHCS secretion with hemorrhage. They proposed an extra-pituitary mechanism regulating adrenal sensitivity to ACTH. The present data support such a possibility and suggest that the kidney plays an important role in this regulation.

It has been proposed recently that angiotensin may be a specific aldosterone stimulating hormone(1,2). The evidence presented here suggests, however, that the action of the kidney in regulation of adrenal cortical secretion is not limited to aldosterone. Slater, Casper, Delea and Bartter(3) were unable to separate aldosterone stimulating and 17 OHCS stimulating effects of angiotensin, even at low doses, though Mulrow and Ganong(2) were able to separate these effects. It seems there must be some reservations concerning the specificity of the role of the kidney in control of aldosterone secretion.

In the burn experiments, 17 OHCS secretion fell to levels not distinguishable from normal after 30 minutes in nephrectomized dogs and 60 minutes in normal dogs. The more rapid return to control levels in nephrectomized dogs may be simply a reflection of the smaller rise after burn rather than a specific effect.

It has been suggested(16) on the basis of experiments on dogs with isolated pituitaries that there may be a humoral factor arising in the hind-brain mediating ACTH release. It is possible that a part of the increase in 17 OHCS secretion observed in those experiments results from extra-pituitary stimulation of the adrenal rather than from extra-hypothalamic stimulation of the pituitary. The present experiments suggest that the kidney may mediate such extra-pituitary stimulation.

Summary. Nephrectomized dogs respond to third degree burns with much less increase in 17 OHCS secretion than do normal dogs. ACTH injection also produces much less increase in 17 OHCS secretion in nephrectomized than in normal dogs. These diminished responses appear to result from decreased adrenal sensitivity to ACTH. It is suggested that the kidney plays an important role in the control of 17 OHCS secretion.

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Acridine Orange Staining of Purified Polyoma Virus.* (26858)

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Recent experimental evidence indicates that polyoma is a DNA virus. Di Mayorca *et al.*(1) extracted an infectious nucleic acid from the virus and demonstrated that infectivity was abolished by treatment with DNAase while RNAase had no effect. The studies

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TABLE I. Staining Properties of Viruses.

Virus preparation nucleic acid	Color developed on acridine orange staining	Nuclease susceptibility DNAase	RNAase	Proteolytic enzyme necessary
Double-stranded animal virus DNA	Yellow green	+	—	+
Double-stranded bacteriophage DNA	" "	+	—	—
RNA	Flame red	—	+	—
Single stranded Φ × 174 DNA	" "	+	—	—

of Smith *et al.*(2) with purified P^{32} labelled virus and the inhibitors of DNA synthesis, aminopterin and bromodeoxyuridine, showed that polyoma virus contained DNA. The cytochemical studies of Allison and Armstrong (3), Williams and Sheinin(4) and Bereczky *et al.*(5) with the fluorochrome acridine orange revealed yellow green nuclear inclusions characteristic of DNA in infected cells. The latter two groups of workers noted that pretreatment with a proteolytic enzyme was necessary before the inclusions became susceptible to DNAase. Although these studies indicate a marked increase in nuclear DNA during polyoma production, they do not show how much of this DNA is actually viral in nature and in what state the viral DNA exists.

Mayor and Hill(6) demonstrated that the fluorochrome acridine orange provided a simple means for differentiating among RNA, double-stranded DNA and single-stranded DNA. The technic is particularly useful in identifying viral nucleic acids. Under carefully controlled conditions of dye concentration and pH the behavior of fixed preparations of animal viruses so far examined may be summarized from their data as in Table I.

The experiment reported here was carried out in an attempt to find where purified polyoma virus fits into this table.

Materials and methods. A stock of plaque purified polyoma virus was provided by Dr. Matilda Benyesh-Melnick, who also carried out all the infectivity and hemagglutination titers by methods already described(7). The titer after 3 passages in mouse embryo tissue culture was 6.3 × 10^7 PFU/ml and the hemagglutinating titer with guinea pig cells was 800/ml(7). Thirty-seven ml of this infected mouse embryo fluid was clarified at 5000 rpm in the Spinco 40 rotor for 30 minutes. The supernatant was removed and spun at 40,000 rpm for 2 hours. The resulting pellet was suspended in approximately 1.2 ml phosphate buffered saline (pH 7.2) by gentle overnight shaking at 4°C. The hemagglutinating titer of this material was 20,480/ml. One ml of this virus suspension was added to 4 ml of a cesium chloride solution to give a final density of 1.28. After centrifugation for 50 hours at 25,000 rpm in the Spinco SW 25 rotor, a sharp band appeared near the middle of the tube. This band was removed in 1 ml of fluid by puncture through the bottom of the tube and after dialysis against phosphate buffered saline had a hemagglutinating titer of 5120/ml.

Results and discussion. If a virus has a buoyant density different from that of contaminating material a purification of the virus can be obtained by isodensity centrifugation. Smith *et al.*(2) have shown that the sharp band appearing in CsCl at an approximate density of 1.29 coincides with maximum activity of the virus. By comparison in CsCl, DNA bands at 1.7, RNA at 2.0 or greater, while most proteins float at a density of 1.25 or less (Schaffer, personal communication). Our bands would appear to contain sufficiently purified polyoma virus. Table II gives results obtained in a second experiment

TABLE II. Assay of Polyoma Virus.*

Virus material	HA titer/ml	PFU/ml
Original virus fluid, 30 ml	160	1.1 × 10^7
Ultracentrifugal pellet resuspended in 1.5 ml	640	3 × 10^7
CsCl band, in 1 ml	80	2.5 × 10^6

* Assays kindly carried out by Dr. Matilda Benyesh-Melnick.

TABLE III. Staining Properties of Poliovirus Φ × 174 and Polyoma.

Fixed virus concentrate	Color developed with AO staining	Nuclease susceptibility DNA-ase	RNA-ase	Proteolytic enzyme necessary
Poliovirus	Flame red	—	+	—
Φ × 174	" "	+	—	—
Polyoma	Yellow green	+	—	+

which included both plaque and hemagglutination assays.

Droplet preparations of this purified polyoma virus suspension were fixed 30 minutes in Carnoy's fluid and stained with 0.01% acridine orange at pH 3.8. The staining technic and relevant enzyme digestion tests have been described in detail(8,6). Simultaneous staining of purified preparations of bacteriophage Φ × 174(6) and poliovirus (8) was carried out as a control. The results are shown in Table III. It would appear that the nucleic acid present in polyoma is DNA, and that in the mature virus particle it exists in the double-stranded form.

It may be argued that a DNA virus replicating in the nucleus could possibly adsorb to its surface sufficient cellular DNA to give a double-stranded color reaction in the standard acridine orange test. To clarify this point, 3 unfixed droplet preparations were first incubated in DNAase (0.01% in veronal buffer) for 10 minutes, 1 hour and 2 hours respectively. They were then rinsed in distilled water, fixed in Carnoy's and stained as above. All preparations developed an equally brilliant yellow green color only slightly less in intensity than the untreated specimens. It woud appear that there is some cellular DNA adsorbed to the virus particle but that this amount is readily and rapidly removed by nuclease digestion leaving the reaction of the intact virus particle essentially unchanged.

Summary. Preparations of polyoma virus purified by CsCl isodensity centrifugation stained yellow green at pH 3.8 with 0.01% acridine orange. Pretreatment with a proteolytic enzyme was necessary before development of the stain could be inhibited by DNAase. RNAase had no effect on similar preparations. These characteristics are consistent with the identification of the nucleic acid of polyoma virus as a double-stranded DNA.

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Vitamin D and Urinary Amino Acid Excretion in the Rat.* (26859)

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An elevated excretion of amino acids in the urine of rachitic children was first observed by Hottinger(1). Freudenberg and Goetz(2) later demonstrated that this could be reversed by a single massive dose of Vit. D. However, Jonxis *et al.*(3), who also reported

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an increase in free and bound urinary amino acids in rachitic children, found that this increased excretion did not return to normal for 3 to 4 weeks after treatment with Vit. D. It was therefore obvious that this effect of the vitamin lags far behind its effect on the calcium and phosphorus levels of plasma and initiation of healing of rickets. Jonxis and Huisman(4) more recently concluded that the increased excretion was not the result of a metabolic disturbance but rather of a defective tubular reabsorption, since plasma amino acid levels were unchanged in rickets. Nevertheless these investigators considered the aminoaciduria to be a primary effect of Vit. D deficiency. Many studies on amino acid excretion have since appeared and have been carefully reviewed elsewhere (Lathem *et al.*(5), Jonxis(6), Evered(7), Harrison and Harrison(8), and Harris(9)).

In this laboratory, the search for a metabolic action of Vit. D has led to a study of amino acid metabolism in rats fed various diets with and without Vit. D. This paper reports results which show clearly that aminoaciduria does not occur in rats deficient in Vit. D.

Methods. Thirty-day-old male albino rats of the Holtzman strain weighing 65-85 g were used in all experiments. They were housed in overhanging wire cages during the first 3-day period and given food and distilled water *ad libitum.* They were then placed in wire metabolism cages and were transferred to separate feeding cages for 3 one-hour periods daily during which time they had access to unlimited quantities of food unless otherwise indicated. Urine collections were made during the 7th-9th, 10th-12th, and 13th-15th days of experiment. Urine was collected under a layer of toluene and feces were retained on wire screens attached to the bottom of the cage. At the end of an experiment, the rats were killed by a blow on the head followed by severing of the carotid artery. When desired, blood was collected in 15 ml centrifuge tubes. Finally, the rachitic state of the rats was estimated by femur ash determinations and by silver nitrate staining of sectioned radii(10).

Free alpha amino nitrogen was measured by the method of Van Slyke *et al.*(11), serum alpha amino nitrogen according to the method of Hamilton and Van Slyke(12), urinary phosphorus by the method of Fiske and Subbarow(13) following digestion with concentrated nitric and perchloric acids, and creatinine according to the method of Owen *et al.*(14). Individual urinary amino acids were identified and determined quantitatively by ion exchange chromatography according to the method of Moore *et al.*(15) with the Beckman/Spinco Amino Acid Analyzer(16).

All rats were fed essentially the basal diet of Bellin and Steenbock(17) which contains D-glucose monohydrate (Cerelose) 67%, steamed egg white 18%, cottonseed oil (Wesson) 10%, roughage (Celluflour) 3%, calcium and phosphorus free salts 2%, and crystalline vitamins. In addition the rats received orally a fat soluble vitamin mixture dissolved in cottonseed oil (Wesson) which supplied 70 μg of β carotene, 875 μg of α tocopherol, and 105 μg of menadione to each rat per week. In each experiment, one-half the rats received orally 75 i.u. of crystalline Vit. D_2 (calciferol) in cottonseed oil per rat every 3 days. The desired levels of calcium and phosphorus in the diet were achieved by addition of $CaCO_3$ and/or an equimolar mixture of KH_2PO_4 and K_2HPO_4 at the expense of the Cerelose. Diet 11 was non-rachitogenic (0.45% Ca and 0.3% P), diets 23 (0.45% Ca and 0.02% P) and 34B (0.02% Ca and 0.02%P) were strongly rachitogenic while diet 11D (0.02% Ca and 0.3% P) produced the low calcium Vit. D deficiency syndrome in agreement with the results of Steenbock and Herting(10). Diet 24 (1.5% Ca and 0.12% P) was another strongly rachitogenic diet in which vitamin-free casein (General Biochemicals Inc.) plus 0.2% L-cystine served as the protein source. Severe rickets was produced by this diet in agreement with the results of Guroff(18).

Results and discussion. Although there have been numerous reports of aminoaciduria in rachitic infants and dogs, studies with rats have been lacking. In the many experiments conducted, we were unable to demonstrate a significant elevation of free alpha amino nitrogen in urine of rachitic rats above

TABLE I. Urinary Free Alpha Amino Nitrogen from Rats Fed Various Diets with and without Vitamin D.*

		mg α-amino nitrogen/100 g rat/3 days				mg α-amino nitrogen/mg creatinine/3 days			
		Ad libitum		Pair fed		*Ad libitum*		Pair fed	
		–D	+D†	–D	+D†	–D	+D†	–D	+D†
Diet 24	(1.5% Ca + .12% P)‡	3.3	3.7	3.1	3.3	.6	.4	.6	.4
" 23	(.45% Ca + .02% P)	6.3	5.3	—	—	.6	.5	—	—
" 34B	(.02% Ca + .02% P)	4.4	4.4	4.7	4.2	.6	.5	.6	.5
" 11D	(.02% Ca + .3% P)	2.1	3.3	1.9	2.1	.3	.4	.3	.3
" 11	(.45% Ca + .3% P)	3.3	4.0	3.1	4.4	.5	.6	.5	.6

* Values are avg from at least 6 animals fed the various diets for 12 days.

† Seventy-five i.u. of vit. D_2 (calciferol) in Wesson oil was given each rat every 3 days.

‡ Protein source was casein plus 0.2% L-cystine.

that in the urine of their Vit. D supplemented controls, in contrast to a previous report(19). Typical results of those obtained are shown in Table I. It is clear that only slight elevations were found in urinary alpha amino nitrogen when the potent rachitogenic diets 23 and 34B were fed. In a further effort to simulate dietary conditions associated with infantile rickets more closely, the protein source was changed to casein plus cystine and calcium level was elevated to insure rachitogenesis. Again, no aminoaciduria was evident (diet 24). These results contrast sharply with those obtained with human rickets in which a 3- to 4-fold elevation of alpha amino nitrogen occurs(20).

The problem of changes in food consumption and animal size is a major consideration in studies such as these despite the expression of amino nitrogen values on an equivalent rat weight basis. To overcome such criticism, rats on the various diets tested were pair-fed with their Vit. D supplemented controls and amino nitrogen excretion was expressed on either a rat weight basis or a urinary creatinine basis. In these experiments (Table I) it was even more evident that aminoaciduria does not occur in rat rickets (diets 23, 34B or 24) or when either a non-rachitogenic (No. 11) or a low Ca (No. 11D) diet is fed. Our results, therefore, do not support the belief of Jonxis *et al.*(4) that aminoaciduria is a primary effect of Vit. D deficiency.

The difference between human and rat rickets with regard to aminoaciduria aroused some interest. The existence of a defective tubular reabsorption of phosphate in rachitic infants and dogs has been known for some time(21). In agreement with others(22), results shown in Table II demonstrate that excessive urinary excretion of phosphate is not found in rachitic rats. It may be a coincidence that rats and humans differ in both amino acid and phosphate reabsorption in rickets; however, a possible relationship between phosphate and amino acid reabsorption in the kidney tubules may provide insight into this problem.

It appeared possible that aminoaciduria in rat rickets might be masked by excessive excretion of a single amino acid which might be unaffected by Vit. D. The amino acids from rachitic and Vit. D supplemented rats were therefore determined qualitatively and quantitatively by ion exchange chromatography. Jonxis and Huisman(23) have reported large losses of serine, threonine, glycine, alanine, histidine, lysine, and bound glutamic acid in urine of rachitic children. Our results shown in Table III clearly reveal that in rat rickets no marked increases occur that might be classified as an aminoaciduria. It should be noted that the relatively large increase in cystine excretion occurred with the diet (No. 24) supplemented with 0.2%

TABLE II. Urinary Phosphorus of Rats Fed Rachitogenic Diets with and without Vitamin D.*

		mg/rat/6 days	
		–D	+D†
Diet 23	(.45% Ca + .02% P)	.8	1.0
" 34B	(.02% Ca + .02% P)	1.0	1.0

* Values are avg from 8 rats fed their respective diets for 12 days.

† Seventy-five i.u. of vit. D_2/rat every 3 days.

TABLE III. Urinary Amino Acids from Rats Fed Rachitogenic Diets with and without Vitamin D.*

	μmoles/100 g rat/3 days			
	Diet 23		Diet 24‡	
	–D	+D†	–D	+D†
Aspartic acid	1.4	3.1	1.4	2.1
Threonine	3.5	3.3	5.0	4.0
Serine, glutamine & asparagine	9.2	5.9	7.9	6.8
Glutamic acid	11.0	12.0	6.9	7.0
Proline, citrulline	4.1	2.9	4.8	3.4
Glycine	14.7	17.5	10.9	11.9
Alanine	6.8	6.7	5.7	6.6
Methionine	2.3	1.6	2.0	1.9
Valine	1.4	.8	1.1	.9
Cystine	—	2.2	47.7	23.2
Isoleucine	1.8	1.3	2.2	1.3
Leucine	1.6	1.6	1.5	1.3
Tyrosine	1.2	.9	1.1	1.0
Phenylalanine	1.2	.9	.8	1.1
Lysine, ornithine	1.7	3.2	4.6	6.0
Histidine	5.7	5.6	1.4	2.2
Arginine	3.0	3.2	2.7	3.2
Methionine sulfoxides	16.0	12.7	3.9	3.6
Total	86.6	85.4	111.6	87.5

* Values represent one analysis of a pooled sample from 10 rats fed their respective diets for 10-12 days.

† Seventy-five i.u. of vit. D_2/rat every 3 days.

‡ Casein plus 0.2% cystine served as protein source.

L cystine. Since no significant effect of Vit. D deficiency on excretion of this amino acid occurred with all the other diets tested, which contain adequate amounts of cystine in the protein form, this was not looked upon as an aminoaciduria associated with rickets. Similar results, not shown, were also obtained with the other diets (Nos. 34B, 11 and 11D) tested.

Summary. Free alpha amino nitrogen as well as individual amino acids have been determined in urine of rats fed various diets with and without Vit. D. In contrast to infantile rickets, no aminoaciduria occurs in Vit. D deficiency whether strongly rachitogenic or non-rachitogenic diets are fed. Furthermore, a rachitogenic casein diet also produced no aminoaciduria.

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Studies on Mechanism of Ascites Produced by Pertussis Vaccine.* (26860)

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Bordetella pertussis vaccine produces a cutaneous hypersensitivity reaction following paw injections(1) and ascites following intraperitoneal injections(2) in the rat. In both cases, the reaction is greatly augmented by prior sensitization with the same vaccine. Prior sensitization also enhances the lethal effects of *B. pertussis* vaccine in mice; the sensitizing ability but not the lethal capacity is destroyed by heat(3,4). The heat stability of the lethal factor has suggested a possible relation to endotoxin(3). In view of these data, it was of interest to determine the effects of heat on the ability of *B. pertussis* vaccine to produce ascites, and to investigate the mechanism of the reaction. The following experiments on the properties of heated *vs.* unheated vaccines and on passive transfer provide no evidence for participation of endotoxin in the genesis of ascites, and in fact, give additional evidence of the immunological nature of this phenomenon.

Methods. Female Wistar-derived rats (Hemlock Hollow Farms), 180-220 g, were fed Purina Laboratory Chow and tap water *ad libitum.* Six-tenths ml *B. pertussis* vaccine‡ containing 36 billion killed phase I organisms was diluted to 3.0 ml with sterile non-pyrogenic saline and injected intraperitoneally through a short-bevelled 21-gauge needle. Heating was accomplished before dilution by suspending the vial in a boiling water bath for one hour. Dose and dilution were the same for sensitizing and challenge injections and for heated and unheated vaccines. Ascitic fluid was measured by draining widely opened peritoneal cavities of exsanguinated rats into graduated cylinders. Omentum, mediastinal and renal lymph nodes and spleen were studied histologically.

Sensitization for passive transfer was accomplished in 3 ways. Ten donors of serum were sensitized with pertussis vaccine according to the method of Rowley *et al.*(1) for donors, and were bled from the heart. Seven donors of mediastinal lymph nodes were given 2 intraperitoneal injections of 3.0 ml diluted pertussis vaccine at an interval of 19 days and were sacrificed 5 days later. Five donors of superficial lymph nodes (popliteal, axillary, elbow) were sensitized intracutaneously in the 4 paws and at 4 flank sites with 1.2 ml of a 1:1 water-in-oil emulsion of undiluted pertussis vaccine and Freund's complete adjuvant; 19 days later they were given 3.0 ml of diluted vaccine intraperitoneally and were sacrificed 5 days later. Lymph node donors were killed by a blow to the head and dissected under sterile conditions. The nodes were minced, suspended in Ringer's solution by cycling between 2 syringes connected by a double-hubbed 18-gauge needle and passed through a sieve. Suspensions from each donor varied in volume from 2.0 to 4.2 ml and were always injected into only one recipient. As controls, several suspensions were heated 20 minutes in a 48°C water bath before being injected(5).

Results. Effects of heating pertussis vaccine. Rats were sensitized and challenged intraperitoneally 12 days later as outlined in Table I. Two injections of unheated pertussis vaccine caused severe ascites in rats sacrificed 5 days after challenge. Two injections of heated vaccine caused no ascites. Cross tests showed that heat destroyed the ability of pertussis vaccine either to sensitize or to elicit ascites. When heated vaccine was followed by unheated vaccine and when unheated vaccine was followed by heated vaccine, rats had no ascites 5 days after chal-

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‡ Donated by Lederle Laboratories through the kindness of Dr. John A. McMillen.

TABLE I. Effect of Heat on Ability of Pertussis Vaccine to Produce Ascites.

Pertussis vaccine		Ascitic fluid (ml)	
1st inj.	2nd inj.	5 days after challenge	1 day after challenge
Unheated	Unheated	26, 24, 22, 19, 6.0, 0	5.3, 4.6, 3.8, 3.2, 1.8
Heated	Heated	0, 0, 0, 0, 0	
"	Unheated	1.0, 0, 0, 0, 0, 0	0, 0, 0, 0, 0
Unheated	Heated	10.0, 0, 0, 0, 0, 0	
" *	" *	0, 0, 0, 0, 0, 0	

* Interval between injections prolonged to 19 days to assure complete subsidence of response to first inj. All other intervals were 12 days.

lenge except for a single anomalous result in each group (Table I).

The first was the occurrence of 1.0 ml of ascitic fluid in one rat pretreated with heated vaccine and challenged with unheated vaccine. This small volume 5 days after challenge could be due to the second injection alone(2). To confirm this, the experiment was repeated but rats were sacrificed 24 hours after challenge, that is, before the challenge injection alone could produce ascites(2). Two injections of unheated vaccine caused ascites but heated vaccine followed by unheated vaccine produced no fluid. Therefore, the anomalous result noted above could not be attributed to sensitization by heated vaccine.

The second aberrant result was found in one rat that had 10.0 ml ascitic fluid after sensitization with unheated pertussis vaccine followed 12 days later by challenge with heated vaccine. This could be due to an exceptionally prolonged and severe response to unheated vaccine used for the first injection. This possibility was confirmed by observation of rats sensitized with unheated vaccine. Twelve days after sensitization an occasional animal showed a swollen abdomen indicative of ascites. This disappeared by 19 days, at which time 6 rats were challenged with heated vaccine. At sacrifice 5 days later these rats had no fluid while ascites was present in all control rats challenged with unheated vaccine. Therefore the aberrant result provided no evidence that heated vaccine could produce ascites in sensitized rats.

Intraperitoneal injections of unheated pertussis vaccine, in addition to eliciting ascites, caused fibrinopurulent peritoneal exudate, inflammation of omentum, hyperplasia and granulomas in spleen and mediastinal and renal lymph nodes, thrombosis of lymph node sinuses, and an increase in body weight averaging 1.2 g per day greater than in untreated controls. None of these changes occurred in rats injected twice with heated vaccine.

Effect of cortisone. Twelve rats were sensitized intraperitoneally with unheated pertussis vaccine. Five of these rats received daily intramuscular injections of 20 mg/kg of cortisone acetate from day of sensitization through day 7 and 10 mg/kg from day 9 through 18. All rats were challenged intraperitoneally with unheated pertussis vaccine on day 19 and sacrificed 5 days later. Rats not treated with cortisone had 16, 16, 11, 8.0, 3.2, 1.4, and 1.0 ml of ascitic fluid (average 8.1 ml. Rats treated with cortisone had only 7.0, 4.8, 0.4, 0.1, and 0.0 ml of fluid (average 2.5 ml). This difference is statistically significant at probability level $p = .05$.

Passive transfer. Each of 6 recipients was given intraperitoneally 3.0 ml serum from actively sensitized donors, and was challenged intraperitoneally 24 hours later with 3.0 ml diluted pertussis vaccine. None had ascites 24 hours after challenge.

Seven recipients were given intraperitoneal injections of living lymph node cells from actively sensitized donors and 5 recipients were given heat-killed lymph node cells. The rats were challenged intraperitoneally with 3.0 ml diluted pertussis vaccine 24 hours later and were sacrificed one day after challenge. None of the rats treated with killed cells had ascites. In contrast, rats sensitized with living cells had 3.2, 2.7, 0.9, 0.5, 0.2, 0.2, and 0.0 ml fluid (average 1.1 ml). Ascites occurred after transfer from either mediastinal or superficial lymph nodes.

Discussion. The results indicate that the fraction(s) of the pertussis organism responsible for sensitization and production of ascites and lesions in omentum, lymph nodes, and spleen are heat-labile and are, therefore, different from the heat-stable lethal factor implicated in the experiments of Kind(3) and Anatolii(4). This suggests that endotoxin is not involved in production of ascites. Sensitization for production of ascites was transferred passively by lymph node cells but not serum from pertussis sensitized donors. It seems likely, therefore, that the ascites phenomenon is analogous to the cutaneous hypersensitivity reaction of Rowley *et al.*(1) because in both cases there are similar responses to 1 and 2 injections of pertussis vaccine(2) and reactivity can be transferred passively with cells from sensitized donors. Cortisone decreased ascites but it is uncertain whether this was due to immunological effects, aberration of electrolyte and water metabolism, or general debility.

Summary. Rats sensitized and challenged intraperitoneally with pertussis vaccine developed severe ascites. However, pertussis vaccine that had been heated for 1 hour at 100°C neither sensitized rats for subsequent challenge nor elicited ascites in adequately sensitized rats. Unheated pertussis vaccine caused lesions in omentum, lymph nodes and spleen, and excessive gain in weight. These were not observed in animals injected with heated vaccine. Sensitization for production of ascites by pertussis vaccine was transferred passively to normal rats by intraperitoneal injection of lymph node cells but not serum obtained from sensitized rats. The results suggest that ascites elicited by pertussis vaccine resembles the delayed type of hypersensitivity and is not related to endotoxin.

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Decrease of Sialic Acid in Epiphyseal Plates of Aminoacetonitrile-Treated Rabbits.* (26861)

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Extensive lesions occur in the connective tissues of animals treated with Lathrogenic agents(1). The epiphyseal plates of young mammals are particularly vulnerable to the action of aminoacetonitriles (AAN). The biochemical nature of the lesion produced with these compounds is poorly understood.

The importance of sialic acid in the connective tissues has been recently emphasized (2,3). The content of sialic acid in the epiphyseal plates of normal and AAN-treated rabbits is here reported.

Method. Twenty 8- to 9-day-old rabbits from 3 different litters were divided into 2 groups. One group of 11 animals received a daily subcutaneous injection of 10 mg AAN during 5 days. The AAN solution was prepared dissolving AAN sulfate neutralized with sodium carbonate in saline solution and stored in a refrigerator at 5°C. Nine control animals received no injections. After 5 days the animals were sacrificed by decapitation and the epiphyseal plates from tibia, femur, and distal radius and ulna were obtained and placed in test tubes in ice. The epiphyseal plates were homogenized in a potter in 0.1 N H_2SO_4 as suggested by Svennerholm(4). The homogenate was hydrolyzed during one hour at 80°C. The hydrolyzate was filtered in a Dowex 50 column and absorbed in Dowex 2 × 8 (analytical grade). The sialic acid was eluted in 1 M acetate buffer pH 4.6 and the eluate was treated with resorcinol copper

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TABLE I. Sialic Acid in Epiphyseal Plate of Normal and Aminoacetonitrile (AAN-Treated Rabbits).

Normal animals			AAN-treated animals		
Tissue tested, mg (f.w.)	Sialic acid, mg	mg/100 mg (f.w.)	Tissue tested, mg (f.w.)	Sialic acid, mg	mg/100 mg
170.2	120.5	70.7	240	133.5	56.2
209	142.4	68.1	153.5	90.5	58.9
132.7	128.5	96.8	203.2	121.0	59.5
146.0	137.5	94.1	198.3	107	53.9
141.8	123.0	86.7	153.4	136.5	89.2
96.2	90.5	94.0	168.7	128.5	76.1
86.5	75.0	86.7	145.4	128.5	88.3
116.1	86.5	74.5	134.4	90.5	67.3
130.0	153.0	117.0	158.3	126.5	79.9
			190.8	102.5	53.7
			121.3	100.0	82.4
Avg ± stand. dev.		87.6 ± 15.28			69.58 ± 13.93

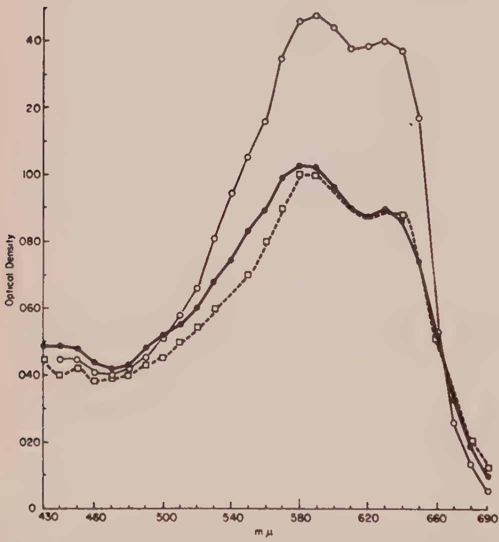

FIG. 1. Absorption curves of chromogens obtained on heating the resorcinol reagent with: Eluate of normal cartilage (mg 146) (———), and of lathyric cartilage (mg 240) (– – – –) after acid hydrolysis (0.1 N H_2SO_4) at 80°C during one hr; standard sialic acid (40 μg) (○····○).

reaction as described previously(2). Optical density was measured with a Beckman's spectrophotometer D.U. The values were compared with the standard curve prepared with sialic acid.§

Results are reported in Table I. A slight decrease in content of sialic acid was observed in the epiphyseal plates of lathyric animals as compared with the normals.

The comparison between the absorption spectra of sialic acid obtained from the epiphyseal plates of normal and lathyric rabbits after resorcinol copper reaction is also reported (Fig. 1).

Summary. Sialic acid is present in the epiphyseal plate of normal animals and is slightly reduced in the epiphyseal plates of AAN-treated rabbits. No qualitative differences were found in the sialic acid of the epiphyseal plates of normal and AAN-treated rabbits tested.

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Effects of Methylandrostenediol on Weight and Cholesterol Content of Adrenals of Cortisone or Thiouracil-Treated Rats.* (26862)

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Methylandrostenediol (MAD) and other androgens will prevent adrenal atrophy in rats treated with cortisone acetate (1, for review). The adrenal-sparing action of androgens has been termed "ACTH-like" because: (a) the effect is exerted directly on adrenal cortex; (b) the hypophysis is not required (1). The term, "ACTH-like," may be inappropriate inasmuch as androgens do not influence adrenal weight in normal rats, and little is known of the functional capacity of androgen-maintained adrenals. In this regard, Saffran and Vogt(2) found that MAD treatment depressed adrenal corticosteroid production by 50% in adult male rats under stressful conditions. A similar depression of adrenal function was observed in rats treated with cortisone or cortisone plus MAD. To the contrary, Dean *et al.*(3) reported a 5-fold decrease in corticoid production of male rats following castration and implied that gonadal steroids are necessary for maximal adrenal secretory capacity. These apparently divergent observations could be partially reconciled if a substrate requisite for corticoidogenesis were androgen-dependent. Cholesterol is a normal precursor of corticoids and changes in adrenal cholesterol have served as useful criteria of ACTH-like activity(4). Thus, cholesterol content may reflect functional capacity of adrenals subjected to other stimuli. Adrenal cholesterol content was, therefore, determined in male and female rats treated with cortisone, MAD and their combination.

Adrenal atrophy, similar to that induced by cortisone, has been observed in rats fed a goiterogen(5, for review). This effect provided a corollary experiment evaluating the "ACTH-like" action of androgens. Accordingly, the effect of MAD on weight and cholesterol content of adrenals were observed in thiouracil-fed rats.

Materials and methods. Male and female rats (Long-Evans strain) of 50 to 70 g body weight were used. Cortisone acetate and methylandrostenediol (aq. susp.) were injected subcutaneously in 1 and 2 mg daily dosages respectively for 7 days. Thiouracil was fed for 20 days at a level of 0.5% in ground fox chow diet.

Adrenals were weighed prior to maceration and extraction with three 2 ml portions of boiling alcohol-acetone (1:1). Cholesterol content of adrenal extracts was determined by the method of Shoenheimer and Sperry as described by Hawk, Oser and Summerson(6). Body weight retarding effects of cortisone acetate dictated that adrenal weights be calculated relative to final body weight.

Results. Cortisone acetate induced adrenal atrophy in male and female rats whereas MAD had no effect alone (Tables I and II). Normal adrenal body weight ratios were, however, observed in rats of both sexes treated concomitantly with cortisone and MAD.

Adrenal cholesterol concentration was significantly depressed to ⅛ the normal value in males and to ⅓ the normal value in female rats treated with cortisone acetate. MAD alone depleted adrenal cholesterol to ¼ the normal level in males. Cholesterol levels in MAD-treated females were even lower than those observed in cortisone-treated females (12% *vs* 33% of normal).

Combined treatment with cortisone acetate and MAD maintained adrenal cholesterol levels at 75% of the normal value in male rats, but the combination of steroids did not prevent adrenal cholesterol decreases in female rats.

When adrenal atrophy was induced by feeding thiouracil to female rats, adrenal cholesterol concentration was not significantly altered. MAD did not prevent thiouracil-

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TABLE I. Influence of Cortisone Acetate and MAD on Adrenal Weight and Cholesterol Concentration in Male Rats.

Treatment	No. rats	Body wt, g	Adrenal wt, mg/100 g	Adrenal cholesterol, %	
				Total	Free
None	8	78 ± 2*	19.3 ± .8	1.66 ± .12	.16 ± .01
Cortisone acetate, 1 mg daily × 7	19	62 ± 3	10.6 ± .5	.25 ± .05	<.01
MAD,† 2 mg daily × 7	15	89 ± 4	18.2 ± .9	.44 ± .04	.10 ± .002
Cortisone acetate, 1 mg + MAD, 2 mg daily × 7	21	67 ± 5	17.6 ± .5	1.09 ± .11	.05 ± .002

* S.E. of mean. † MAD = methylandrostenediol.

TABLE II. Influence of Cortisone Acetate, MAD and Thiouracil on Adrenal Weight and Cholesterol Concentration in Female Rats.

Treatment	No. rats	Body wt, g	Adrenal wt, mg/100 g	Adrenal cholesterol, %	
				Total	Free
None	14	104 ± 3*	19.5 ± .9	3.49 ± .32	.26 ± .02
Cortisone acetate, 1 mg daily × 7	13	68 ± 5	10.9 ± .7	1.20 ± .37	.07 ± .03
MAD, 2 mg daily × 7	8	105 ± 4	17.4 ± .8	.40 ± .08	.06 ± .01
Cortisone acetate, 1 mg + MAD, 2 mg daily × 7	13	64 ± 4	17.1 ± 1.0	1.34 ± .22	.05 ± .01
0.5% thiouracil in diet × 20	5	82 ± 5	12.4 ± .2	3.18 ± .30	.21 ± .06
Idem + 2 mg MAD × 20	5	93 ± 4	13.6 ± .8	1.01 ± .22	.17 ± .02

* S.E. of mean.

induced adrenal atrophy, but did deplete adrenal cholesterol by 67%.

Discussion. These data confirm and extend the histochemical observations of Gaunt *et al.* (1), who noted a loss of Schultz-positive (cholesterol) material in adrenals of male rats treated with cortisone or MAD. Present data suggest a sex difference in the effects of an androgen on adrenal function, since adrenal cholesterol was (partially) protected by MAD in cortisone-treated males, but not in cortisone-treated female rats. Also, MAD alone depleted cholesterol to a greater extent than did cortisone alone, in female rats, while inducing a lesser, but significant cholesterol loss from adrenals of male rats. Full significance of these effects awaits experimental evidence of the corticoidogenic capacity of adrenal glands of rats treated with cortisone, MAD, or their combination. Van der Vies (7) found that adrenals obtained from hydrocortisone-treated rats produced lesser amounts of corticoids when incubated *in vitro* than did adrenals of normal rats. Saffran and Vogt(2) observed *in vivo* a 50% reduction in adrenal corticoid production by male rats pre-treated with MAD, cortisone or their combination. Reduced corticoid production reported in MAD- or cortisone-treated rats correlates well with current data on cholesterol. Thus, severe depletion of cholesterol, a major corticosteroid precursor, may be responsible for reduced corticoidogenesis(2).

Less easily explained is the near-normal cholesterol level in adrenals of male rats receiving MAD and cortisone acetate concomitantly, and the observations of Saffran and Vogt(2) that animals so treated exhibit a reduced corticoid output. However, Saffran and Vogt did not obtain the expected adrenal weight maintaining effect of MAD in cortisone-treated rats, possible because they used intact adult males instead of immature rats (1).

MAD did not protect against thiouracil-induced adrenal weight loss. Since MAD is known to maintain essentially normal adrenal weights in hypophysectomized rats(1) the data suggest that thiouracil may reduce adrenal weight by some extra-pituitary mechanism(5).

Summary. Cortisone acetate depressed adrenal weight and reduced adrenal cholesterol concentration in male and female rats. Methylandrostenediol (MAD) depleted adrenal cholesterol in both sexes while not influencing adrenal weight. When cortisone acetate and MAD were injected concomitantly, normal adrenal weights were observed in male and female rats. However, adrenal cholesterol content was protected by MAD only in cortisone-treated males. MAD did not prevent adrenal weight loss due to thiouracil, but did deplete cholesterol by 67%.

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Plasma Thromboplastin Antecedent Levels in Patients Receiving Coumarin Anticoagulants and in Patients with Laennec's Cirrhosis.* (26863)

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Naeye reported(1) that plasma thromboplastin antecedent (PTA) levels were depressed by Dicumarol (bishydroxycoumarin). However, Waaler later found(2) a normal formation of activation product (which requires PTA) in plasma from 10 patients receiving long term therapy with Dicumarol or phenylindanedione. In addition, a recent preliminary report by Rodermund, Schneider and Egli(3) describes a normal formation of activation product in subjects given Marcoumar for 10 days.

Naeye also reported(1) that PTA levels fell in liver disease. Other reports of the quantitative measurement of PTA activity in liver disease have not been found.

A specific one-stage assay for PTA has been reported from this laboratory recently(4). This test has been used to measure PTA levels in a group of patients receiving coumarin anticoagulants and in another group of patients with advanced Laennec's cirrhosis.

Methods. Blood for testing was drawn with Monocote (Armour and Co.) coated needles and silicone (Dri-film, G.E. SC-87) coated syringes. Nine parts of blood were added to 1 part of anticoagulant (made by mixing 2 parts of 0.1 M citric acid and 3 parts of 0.1 M sodium citrate) in a polyethylene tube. Platelet-poor plasma was prepared by centrifuging for 20 minutes at 14,500 g at 4°C and was tested within about 3 hours after the blood was drawn.

The technic and standardization of the quantitative assay for PTA have been reported elsewhere(4). The clotting system consists of: 0.1 ml of a "cephalin" reagent diluted in a suspension of kaolin powder, 0.1 ml of hereditary PTA deficiency substrate plasma, 0.1 ml of a 1/5 or a 1/10 dilution of the test plasma, and 0.1 ml of 35 mM calcium chloride solution. Neither the substrate plasma nor the test plasma contacts an activating surface prior to its addition to the clotting mixture.

The assay measures PTA activity accurately at levels above about 15% of a standard reference plasma. For example, the 90% confidence limits for a single observed value of 50% PTA vary between 42 and 60% of the reference plasma.

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TABLE I. PTA Levels in Patients Receiving Coumarin Anticoagulants.

Subject	PTA level, %	Prothrombin time (P & P), %
Dicumarol pts.		
A.H.	80	10
R.R.	88	7
M.L.	140	15
J.F.	160	10
L.A.	128	18
Coumadin pts.		
T.	100	29
B.	108	22
H.	84	9
W.	120	30
U.	100	17
L.	92	22
Mc.	120	22
O.	88	22
39 normal subjects: Mean 97%, range 63-136%		

Prothrombin times were measured by the P&P technic of Owren and Aas(5).

Results. PTA levels and prothrombin times of 5 patients receiving Dicumarol and 8 patients receiving Coumadin (sodium warfarin) are listed in Table I. All of the patients had received anticoagulants for at least 2 weeks; several had received anticoagulant therapy for many weeks. As can be seen from the prothrombin times, all of the patients were receiving therapeutic amounts of anticoagulant. PTA levels were normal in every patient.

PTA levels were measured on 3 occasions in an additional patient who had received phenylindanedione for many months. His prothrombin time was consistently below 30%; his PTA levels were 68, 88, and 88%, respectively. Thus, no evidence was found that coumarin or coumarin-like anticoagulants interfere with synthesis of PTA.

TABLE II. PTA Levels in Advanced Laennec's Cirrhosis.

Patient	PTA level, %	Prothrombin time (P & P), %
Roz.	87	94
M.	52	63
So.	59	56
Rod.	52	45
Ra.	49	44
Sm.	48	50
W.	47	55
MaG.	35	33
E	27	35

PTA levels and prothrombin times of 9 patients with advanced Laennec's cirrhosis are summarized in Table II. These patients had decompensated disease with jaundice or ascites or both. Their PTA levels were depressed; the degree of depression closely paralleled the value for prothrombin time. Two of the patients, MaG. and E., had PTA levels of about 30%, which is thought to be the minimum PTA level for effective hemostasis (6).

Discussion. These data, obtained with a specific assay for PTA, confirm the findings of Waaler(2) and of Rodermund and coworkers(3), obtained with less specific technics. They do not agree with the observations of Naeye(1). It appears that the coumarin and coumarin-like anticoagulants do not affect PTA. Therefore, one may conclude that PTA synthesis does not require Vit. K.

The low PTA levels found in patients with chronic parenchymal liver disease confirm the findings of Naeye(1). One may strongly suspect that the liver cell is the site of synthesis of PTA. It would appear that the PTA level of the patient with Laennec's cirrhosis is predictable from his prothrombin time value.

Summary. 1. Plasma thromboplastin antecedent (PTA) levels are normal in patients receiving Dicumarol, Coumadin, or phenylindanedione. 2. PTA levels are depressed in patients with Laennec's cirrhosis. Degree of depression parallels prothrombin time value.

I wish to thank Miss Mary Jane Patch and Miss Sara Beth Ames for valuable technical assistance.

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Agents Providing Nonenzymic Prothrombin Activation. (26864)

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An unusual nonenzymic prothrombin activation was firmly established by demonstrating thrombin formation in mixtures of synthetic poly-L-lysine (polyLYS) and homogeneous horse prothrombin(1). Reaction characteristics were a high, optimum *weight* ratio of synthetic protein to prothrombin, a low temperature optimum, high sensitivity to ionic strength changes, and insensitivity to diisopropylphosphofluoridate (DFP). Several basic materials now substitute for the polyLYS, including synthetic poly-L-ornithine (polyORN) and polymyxin B. The prothrombin activation in protamine sulfate solution, for which an autocatalytic mechanism was suggested(2), has several characteristics of this form of nonenzymic reaction.

Nonenzymic prothrombin activation is inhibited by heparin, poly-L-glutamic acid (polyGLU), and poly-L-aspartic acid (polyASP).

Materials and methods. Horse prothrombin isolated from citrated horse plasma in 60% yield was thrombin-free and immunochemically and physicochemically homogeneous after equilibrium ion-exchange chromatography(3,4). Synthetic tri-L-lysine (triLYS) was kindly donated by Dr. Bernard Erlanger of Columbia University. Poly-L-histidine (polyHIS), approximately 6,000 molecular weight, was contributed by Dr. Gerald Fasman, Children's Cancer Research Foundation, Boston, Mass., and Dr. F. R. N. Gurd, School of Medicine, Indiana University. Synthetic polyLYS, polyORN, polyGLU, and polyASP were commercial products. Antibiotics, protamine, and heparin were commercial preparations free of solubilizers and extraneous salts.

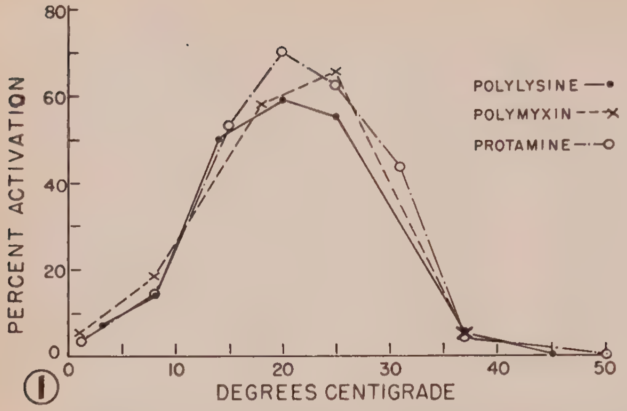
Prothrombin and thrombin were titered according to the methods of Ware and Seegers(5), using polyethylene tubes to dilute thrombin samples.

Substitutes for polyLYS in the nonenzymic prothrombin activation were screened by testing 4.5 to 5.0 mg of each with 3.0 to 3.5 mg samples of horse prothrombin in 2.0 ml of 0.08 *M* Tris buffer, pH 8.0, at 25°C. Reactions were followed by thrombin and prothrombin titrations; the degree of activation is recorded in Table I. Controls included equivalent mixtures of preformed thrombin with each test material. Inhibition of the nonenzymic reaction by heparin, polyGLU, and polyASP was tested by adding 3 mg of each to systems containing polyLYS and prothrombin in the above proportions (Table I). The optimum activation temperatures of prothrombin-polymyxin B and prothrombin-protamine mixtures were determined for comparison with the synthetic polyLYS-prothrombin reaction(1) (Fig. 1).

To rule out enzymic contamination, samples of an aqueous protamine sulfate solution were heated 15 minutes at 100°C or incubated 30 minutes in 5×10^{-3} *M* DFP. Following dialysis, these samples and the original solution activated horse prothrombin at pH 7.5. Subsequently, 3.2 mg of horse pro-

TABLE I. Nonenzymic Prothrombin Activation.

Substance added	% activation 2 hr	 8 hr
PolyLYS	74	83
PolyORN	65	75
Polymyxin	65	70
Protamine	60	70
Neomycin	6	16
Streptomycin	3	10
PolyLYS + heparin	0	0
PolyLYS + polyGLU	0	0
PolyLYS + polyASP	0	0
TriLYS	0	0
PolyHIS	0	0
PolyGLU	0	0
PolyASP	0	0
Bacitracin, Novobiocin, Vancomycin, Ristocetin, Erythromycin, Amphotericin B, Nystatin	0	0



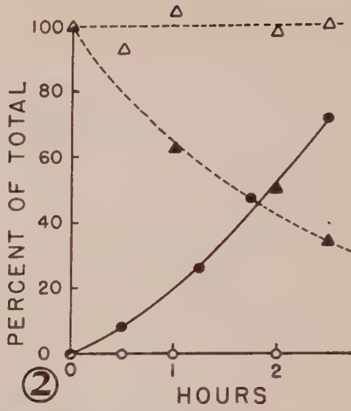


FIG. 1. Effect of temperature on nonenzymic prothrombin activation. With polymyxin and protamine for 2 hr and with polyLYS for 1.5 hr.

FIG. 2. Per cent thrombin (spheres) and prothrombin (triangles) in the following systems: Prothr. + protamine (●——●); prothr. + protamine + 3.8 × 10^{-3} M DFP (○——○) and (▲- - -▲); and prothr. + saline + DFP (△- - -△).

thrombin and 6.0-mg samples of heat-treated or DFP-treated and dialyzed protamine were combined in 2.0 ml of 0.05 *M* imidazole buffer, pH 7.5, which contained 3.8 × 10^{-3} *M* DFP. DFP was omitted from an otherwise identical reaction mixture, and saline was substituted for protamine in another control. Prothrombin consumption in the DFP-containing systems and thrombin formation in the DFP-free-systems are plotted in Fig. 2.

Results. At the optimum conditions characterizing nonenzymic polyLYS-prothrombin activation, polyORN, polymyxin B, and protamine also activate the zymogen. Neomycin and streptomycin may also function to a slight degree in the same process. Synthetic triLYS, polyHIS, polyGLU, and polyASP, as well as a variety of peptide and nonpeptide antibiotics, failed to activate horse prothrombin. Heparin, polyGLU, and polyASP completely inhibited the activation by polyLYS (Table I). Optimum temperatures for the polymyxin B and protamine reactions were identical to the characteristic low (15° to 28°C) optimum of the polyLYS-prothrombin activation (Fig. 1).

The prothrombin activating property of protamine was stable on pretreatment at 100°C or with DFP. Prothrombin consumption in the prothrombin-protamine-DFP mixtures occurred concomitantly with thrombin formation in the DFP-free systems suggesting that the reaction itself is resistant to DFP. No measurable thrombin appeared in the reactions containing DFP (Fig. 2).

In proper proportions for nonenzymic activation the mixtures of prothrombin with polyLYS, polyORN, and protamine form heavy coprecipitates which do not affect the activation reactions. No such precipitates form at optimum polymyxin B-prothrombin mixtures.

Discussion. Thrombin formation in polyORN (free δ-NH_2 groups) and in polymyxin B (5 free γ-NH_2 groups per mole (6-8)) excludes the absolute specificity for the ε-NH_2 groups of polyLYS in nonenzymic prothrombin activation. However, inhibition of the reaction with the polyanions (heparin, polyGLU, and polyASP) supports requirement for primary amino groups and/or strong positive charges. Prothrombin activation in polyLYS but not with an equal weight of triLYS suggests that the reaction requires proper spatial conformation of the free amino groups and/or positive charges.

Prothrombin activation in protamine solution resembles the polyLYS-prothrombin system in having a low temperature optimum and similar kinetics of prothrombin consumption and thrombin formation in presence and absence of DFP(1). Inhibition of thrombin as it forms in systems containing DFP tends to rule out an autocatalytic process. Unless extraordinarily resistant, a prothrombin activating enzyme is an improbable contaminant of the heterogeneous protamine because activity resists 100°C heat and DFP treatment.

The ability of polymyxin to activate prothrombin by the nonenzymic process may be a toxic mechanism of this drug as well as of some other antibiotics. Interestingly, by simply adding heparin to the antibiotics, Higginbotham(9) diminished the lethal effect in mice of polymyxin, neomycin, viomycin, and dihydrostreptomycin while not affecting neomycin antibiotic properties.

If a model for physiologic processes, the mechanism of nonenzymic prothrombin activation may *initiate* blood coagulation at bleeding sites(10). Similarities between this reaction and the action of the combined calcium ion, tissue thromboplastin, and accessory factors include insensitivity to and progression in DFP, whereas autocatalytic activation is inhibited by small amounts of DFP (1,11). If not a physiologic process, nonenzymic prothrombin activation still remains a potential *nonspecific* contributor to *in vitro* coagulation experiments, particularly those involving purified protein reactants in high concentration. Extreme care should be exercised in controlling, ruling out, or including this reaction when interpreting coagulation experiments.

Summary. Synthetic polyORN, polymyxin B, and protamine activate horse prothrombin by processes resembling the nonenzymic activation with polyLYS. Neomycin and streptomycin may function to a slight degree in the same mechanism. Nonenzymic prothrombin activation with polyLYS is inhibited by heparin, polyGLU, and polyASP. The capacity of protamine preparations to activate prothrombin is stable to heat, DFP, and dialysis. Prothrombin consumption in prothrombin-protamine-DFP mixtures is concomitant with thrombin formation in DFP-free systems. Nonenzymic prothrombin activation may be associated with some drug toxicities as well as with physiologic hemostasis.

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Cholesterol in Vitamin B_{12}-Deficient Chick Embryo. (26865)

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Vitamin B_{12} has been implicated in cholesterol metabolism in various species. The blood cholesterol level was found to be abnormally low in pernicious anemia patients in relapse(1,2,3). After effective liver therapy, owing presumably to the action of the Vit. B_{12} present, cholesterol level increased. Forbes and Patterson(4) showed that the livers of Vit. B_{12}-deficient rats had higher levels of cholesterol than the control livers. Hsu

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TABLE I. Cholesterol Content of Developing Chick Embryos from Vitamin B_{12}-Deficient and Normal Eggs.[*]

	Tissue	Days of incubation	No. samples[†]	Avg dry wt (g)	Cholesterol		Total (mg/tissue)[‡]
					Free	Ester	
Normal	Egg[§]	0	16	14.42	256	14	270
	Embryo	12	—[‖]	—	—	—	—
		15	7 (21)	1.84	16.3	7.4	23.7
		18	7 (21)	4.21	34.7	16.4	51.1
		21	7 (19)	6.74	39.9	48.6	88.5
	Yolk sac[¶]	12	4 (20)	14.73	268	7	275
		15	7 (21)	11.96	213	24	237
		18	7 (21)	9.33	151	33	184
		21	7 (19)	4.88	89	30	119
Vit. B_{12}-deficient	Egg	0	16	14.27	258	7	265
	Embryo	12	—[‖]	—	—	—	—
		15	7 (22)	1.65	16.7	6.6	23.3
		18	7 (20)	3.55	30.4	16.7	47.1
		21	6 (16)	5.86	37.1	51.6	88.7
	Yolk sac	12	4 (20)	13.89	250	3	253
		15	7 (22)	11.87	190	45	235
		18	7 (20)	9.70	137	59	196
		21	6 (16)	6.41	87	51	138

[*] Liver and 0.02-0.1 ml blood were removed from each embryo for other analyses, cholesterol was not determined on these samples. Cholesterol contents of livers from normal 21-day-old embryos were: free, 2.8 mg; esterified, 27 mg; and total, 29.8 mg/liver.

[†] Individual eggs were assayed and composite samples of embryos and yolk sacs. Numbers in parentheses represent total No. of embryos and yolk sacs.

[‡] All values are on a dry wt basis.

[§] 8 eggs were collected after 5 wk on experimental diets, and 8 eggs after 9-10 wk on diets. There was no difference in results obtained, so values have been averaged.

[‖] Insufficient tissue left for analyses.

[¶] Sample consists of egg contents except the embryo.

and Chow(5) reported increased amounts of cholesterol in sera of B_{12}-deficient adult rats and chicks, and in adrenals of deficient young rats and chicks. In view of these conflicting data, it was thought appropriate to report the results obtained on B_{12}-deficient chick embryos which were produced for an extensive study of the effect of a Vit. B_{12} deficiency on lipid metabolism in the developing embryo (6,7,8).

Procedure. Vit. B_{12}-deficient and control embryos and yolk sacs[†] were obtained by methods previously described(8). The lyophilized samples were extracted with acetone-ethanol (1:1) and total and free cholesterol determined by the method of Sperry and Webb(9) using aliquots of the extract.

Results. Table I shows cholesterol contents, free, esterified and total, in the chick embryo and yolk sac as the embryo develops. There is no statistical difference between amount of cholesterol in the Vit. B_{12}-deficient tissues and that in the corresponding control tissues. Since the deficient embryo was smaller than the control embryo at 18 and 21 days of age, concentration of cholesterol on a per gram basis is slightly higher in the deficient tissue. The deficient eggs were definitely low in B_{12}[‡] averaging 65 mμg/egg *vs.* 800 mμg for the control egg.

Almost all of the cholesterol in the unincubated egg is free cholesterol, as others have shown(10-13). As incubation proceeds beyond the 12th day, the free cholesterol in the yolk decreased markedly and the level of esterified cholesterol increased somewhat. In the embryo an increase in both free and esterified cholesterol occurred from the 15th day on.

At 21 days there was a 15-25% decrease in total cholesterol in the egg in both control and deficient tissue. The "loss" seemed to be in the ester fraction of the embryo. At 21 days the cholesterol esters are reported to be much increased in the embryo(13). The reason for this discrepancy is not apparent. However, the livers had been removed from all of the embryos, and since a large portion of the esterified cholesterol occurs in the liver, and livers of the 21-day-old embryos were much larger than the others, some cholesterol may have been unaccounted for. This seems not to be the case in samples other than at 21 days. The majority of these values obtained agree well with those in the literature(13).

[†] All contents of the egg except the embryo.

[‡] Determined by *Ochromonas malhamensis* assay, see reference(8).

Summary. The cholesterol content (free and esterified) of the chicken egg during incubation did not differ from that of its Vit. B_{12}-deficient counterpart. Under the conditions of this experiment where a marked B_{12} deficiency existed, Vit. B_{12} has no effect on cholesterol status of the developing chick embryo.

The technical assistance of Catherine Kostuk is gratefully acknowledged.

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Brain 5-Hydroxytryptamine in Ozone-Exposed Rats.* (26866)

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A previous communication(1) discussed the elevation of lung 5-hydroxytryptamine (5-HT) content in rats subjected to acute ozone exposure. In studying potential mechanisms of this increase, the possibility that non-pulmonary tissues might be sources of lung 5-HT was considered, and brain 5-HT levels of ozone-exposed rats were determined. The following describes the results.

Materials and methods. Male albino Wistar rats (225-275 g) were exposed for 4 hours to ozone, 6 parts per million (p.p.m.) by volume in air, as previously described(1). Control animals were handled similarly but exposed to air alone. Control and exposed groups consisted of 22 animals each. After exposure the animals were anesthetized with pentobarbital sodium. Brain stems were severed at the foramen magnum, the brains removed, weighed, and assayed for 5-HT spectrophotofluorometrically in an Aminco-Bowman spectrophotofluorometer by the method of Bogdanski, *et al.*(2). Brain protein was determined by the method of Lowry, *et al.* (3).

Results. Exposure of rats to 6 p.p.m. ozone by volume for 4 hours resulted in a significant decrease in brain 5-HT from a mean control value of 0.63 ± 0.14 μg/g to a mean of 0.39 ± 0.10 for the ozone-exposed group. In view of the observations by Bourdillon *et al.*(4) that inhalation of 5% CO_2 increased Na^{24} entry into the cerebrospinal fluid, the

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TABLE I. Mean Brain Weight, Protein, and 5-HT of Normal Male Rats Exposed for 4 Hours to Air Alone and to 6 p.p.m. Ozone.

Group	g brain/100 g body wt	g protein/100 g brain	μg 5-HT/g brain
Control	.72 ± .06	14.8 ± 1.4	.63 ± .14
Ozone	.78 ± .07*	13.7 ± 1.3	.39 ± .10†

* P <.01. † P <.001.

possibility was considered that hypercapnia resulting from pulmonary edema might cause brain edema and that this might produce an apparent decrease in brain 5-HT concentration by dilution. That some brain edema may occur is indicated by a slight increase in mean brain weight per 100 g body weight of the ozone-exposed group; however, no significant change is noted in brain protein content. The increase in brain weight (8%) is not sufficient to account for the marked decrease (38%) in brain 5-HT. These results are summarized in Table I.

Discussion. Brain 5-HT of ozone-exposed rats is lost and not merely diluted by the development of brain edema. This finding presents a series of problems. First is that of the nature of the 5-HT release from the brain and whether it is similar to that induced by reserpine. Next is the question of the fate of the released 5-HT, *i.e.*, if it is destroyed immediately in the brain or if it may be transported to the lung thereby accounting for part of the increase in this organ. If the later, the transport mechanism should be studied. Finally, the depression observed in ozone-exposed rats may be related, in part at least, to the loss of brain 5-HT; this should be clarified. These problems are now under investigation, and it is hoped that answers will soon be available.

Summary. A significant decrease in brain 5-HT of rats exposed to 6 p.p.m. ozone for 4 hours has been demonstrated. This was an actual loss of 5-HT from the brain, not merely an apparent decrease resulting from dilution by brain edema although some edema occurred.

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Effect of Homologous Blood Components and Heterologous Lysed Red Blood Cells on Dog Renal Dynamics.* (26867)

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Peripheral intravenous infusion of homologous lysed red blood cells produced renal vasoconstriction in dogs(1). These changes in renal dynamics varied with rate of increase in plasma hemoglobin concentration rather than level acheived. On the contrary, no significant renal response occurred when the injection was made into the portal vein(2). Since rates of hemoglobin infusions and plasma hemoglobin concentrations were comparable in the peripheral infusion and portal infusion studies, it was thought possible that passage through the liver altered the effects of these solutions on glomerular filtration rate and effective renal plasma flow. In these experiments, lysed washed dog red blood cells were used, containing red blood cell stroma and other materials of the red blood cells in

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addition to hemoglobin. Although the vasoconstriction might be caused by hemoglobin alone, the possible effects of other red cell components need investigation and are the subject of the present report. In addition, since these components vary from species to species, it was thought advisable to study the effects of heterologous lysed red blood cell infusions when given by peripheral, as well as, portal infusion.

Method. Female mongrel dogs were prepared and measurements of GFR (glomerular filtration rate) and ERPF (effective renal plasma flow) with creatinine and para-aminohippurate, respectively, were done as previously described(1). Femoral artery blood pressures were recorded directly utilizing a Statham gauge and a Sanborn Twin Viso recorder.

A protein free solution was prepared aseptically from dog's red blood cells. The whole blood was spun and washed 3 times with saline in a refrigerated centrifuge. The protein was then precipitated from the red blood cells with alcohol, using 4 cc 95% ethyl alcohol to 5 cc of red blood cells. The precipitate was separated from the solution either by centrifugation or with a Buchner funnel. The solution was Seitz filtered and frozen until used.

For preparation of red blood cell stroma, the washed red cells were lysed in cold distilled water and the pH adjusted to 5.8 with CO_2. The stroma was separated from the solution using a Sharple's centrifuge, washed with 250 cc distilled water, resuspended in a minimal amount of distilled water and frozen.

To prepare the partially purified hemoglobin solution, the washed cells were brought to a pH of 5.5 (0.1 N HCl) with constant agitation. The precipitate was separated by centrifugation and the supernate slowly adjusted to a pH of 7.4 with 1 N NAOH. The solution was Seitz filtered and frozen.

Whole blood was collected with aseptic technics from the sheep, horse, and cow and 50,000 units of aqueous penicillin and 50 mg streptomycin were added to each pint of blood. Each bottle was cultured and discarded if contaminated. The human blood was collected in the hospital blood bank. Preparation of the lysed red blood cells, determination of plasma and urine hemoglobin, and peripheral vein and portal vein infusions were performed as previously reported(1,2).

TABLE I. Effect of Intravenous Infusion of Blood Components on GFR and ERPF in Dogs.*

No. of dogs		I.V. infusion I	I.V. infusion II	Post-infusion I	Post-infusion II
	Protein-free filtrate				
3	GFR	−5	−7	−2	−1
	ERPF	−7	−7	+2	+3
	Red blood cell stroma				
4	GFR	−1	−7	−15	−2
	ERPF	+1	−12	−18	+1
	Partially purified hemoglobin				
4	GFR	−20	−36	+1	−4
	ERPF	−21	−44	+25	+46

* Figures reported as % of normal control.

Results. Protein free filtrate. The effect of intravenous infusion of a protein free filtrate of red blood cells is recorded in Table I. The slight decrease in GFR and ERPF during actual infusion is insignificant. No significant change occurred in urine volume or blood pressure.

Red blood cell stroma. The effects of the hemoglobin free red blood cell stroma are recorded in Table I. No effect was seen in the first infusion period and an insignificant decrease in GFR and ERPF in the second infusion period. A slightly greater decrease in both GFR and ERPF occurred in the first post-infusion period. No significant changes in urine volume or blood pressure were observed. The stroma solution contained Na 105 mEq. per liter, Cl 104 mEq. per liter, and K 0.3 mEq. per liter. Total protein measured 0.012 g %.

Purified hemoglobin. Partially purified hemoglobin solution (Table I) caused a significant decrease in GFR, ERPF and urine flow in both infusion periods. Rate of hemoglobin infusion was 153 mg/kg/min in the first period and 145 mg/kg/min in the second period. Plasma hemoglobin levels during the first and second infusion periods were 2.0 and 3.4 g % respectively and during the first and second post-infusion periods were 3.4 and 3.2 g % respectively. Blood pressure remained stable during the experiments. The hemoglobin solution contained Na 98 mEq.

TABLE II. Effect on Dog Renal Dynamics of Heterologous Lysed Red Blood Cells during Brachial Vein Infusion in 10 Dogs.*

	Brachial infusion		Post-infusion	
	Hb I	Hb II	I	II
GFR	–64	–57	–26	– 6
ERPF	–60	–54	+1	+28
Plasma Hb, g %	1.9	3.3	3.1	2.9
mg Hb/kg/min.	132	126	0	0

* GFR and ERPF reported as % of normal control.

per liter, Cl 75 mEq. per liter, and K 3 m Eq. per liter. The methemoglobin component averaged 18% of the total with a range from 5% to 25%.

Brachial vein infusion of heterologous lysed red blood cells. Table II is a summary of the effects of the brachial infusion group which consisted of 4 dogs in the human and 2 dogs each in the cow, horse and sheep lysed cell infusion experiments. All experiments are averaged as a group since the effects on renal dynamics were the same with all sources of heterologous blood.

Significant decreases in GFR and ERPF occurred during the infusion (Hb I and Hb II) of the heterologous lysed red blood cells in all experiments. Urine flows were uniformly depressed during infusion periods followed by post infusion diuresis as noted with homologous lysed red cell infusion.

Arterial blood pressure remained relatively stable throughout the experiments except in 2 instances. In one experiment using cow red cell lysate the pressure dropped from a control level of 102/65 to 77/45 over a 15-minute period and returned to slightly above normal upon completion of the infusion. In one experiment using horse red cell lysate the pressure dropped from 120/80 to 45/25 within 3 minutes and returned to 120/85 within 7 minutes after starting the infusion. An insignificant rise above control levels frequently appeared during post-infusion periods.

In all experiments with either homologous or heterologous red blood cell lysates the GFR and ERPF returned to normal levels in the first post-infusion period with one exception. In the first post-infusion period (Post Hb I) of the cow red cell lysate experiments, the GFR and ERPF were greatly decreased and in the second post-infusion period (Post Hb II) the GFR was still significantly decreased. As noted above in one of these dogs blood pressure demonstrated a moderate fall, and both manifested a marked increase in pulse, respiration, and developed purpuric lesions over the entire body. Oozing of blood occurred at the cut-down site where the arterial needle was inserted. Body temperature, recorded by a rectal thermometer, remained normal.

Portal vein infusion of heterologous lysed red blood cells. Table III is a summary of the effects of portal vein infusion. There were 3 dogs in the human and 2 dogs each in the cow, horse, and sheep lysed red blood cell experiments. A significant decrease in GFR and ERPF occurred in all experiments during portal infusion periods (Hb I and Hb II). A moderate decrease in urine flow followed by a post-infusion diuresis was noted as in previous experiments.

During the first infusion period of human lysed red cells blood pressure fell to shock level (46 mm Hg mean pressure) for 4 minutes in one experiment and to 58 mm Hg mean pressure for 2 minutes in another experiment. A similar drop was noted in one instance using cow red cell lysate in which the pressure fell to 70/37 within 3 minutes and returned to normal within 11 minutes. In one experiment using horse red cell lysate the pressure dropped from 133/92 to 70/56 in 4 minutes and gradually returned to 136/100 in 15 minutes after start of infusion. One dog's blood pressure, while receiving sheep red cell lysate, fell from the control level of 140/92 to 70/62 within 6 minutes and back to control level within 12 minutes of start of infusion. The rapid pulse and respir-

TABLE III. Effect on Renal Dynamics of Heterologous Lysed Red Blood Cells during Portal Vein Infusion in 9 Dogs.*

	Portal infusion		Post-infusion	
	Hb I	Hb II	I	II
GFR	–57	–53	– 3	+ 1
ERPF	–64	–49	+33	+35
Plasma Hb, g %	1.9	2.8	2.7	2.6
mg Hb/kg/min.	118	107	0	0

* GFR and ERPF reported as % of normal control.

ation, bleeding tendency, and decreased GFR and ERPF in the post-infusion period noted with brachial infusion of cow's lysed red blood cells did not occur in the portal infusion experiments. The blood used in both groups of experiments came from the same donor cow.

Since the human red blood cell has a high potassium content, potassium levels in the lysed cell solution were determined and found to be 21.5 mEq. per liter. Average potassium of the dogs' control serum was 3 m Eq. per liter and average levels for the periods Hb I, Hb II, Post Hb I and Post Hb II were 4.5, 5.5, 5.3, and 4.9 mEq. per liter respectively. As a result of this rise in serum potassium, it was felt necessary to test normal dogs' GFR and ERPF response to increasing levels of serum potassium. Control GFR and ERPF were determined in 3 normal mongrel dogs followed immediately by slow intravenous infusion of a potassium chloride solution containing 184 mEq. per liter. No decreases in GFR and ERPF were noted until serum potassium rose above 7.5 mEq. per liter. Thus, the increase in serum potassium noted, when human lysed cells were infused, was not the cause of the decreased GFR and ERPF.

Discussion. A previous report demonstrated that peripheral vein infusion of homologous lysed red blood cells in dogs caused an acute renal vasoconstriction which could be reversed or prevented with a vasodilator drug, magnesium sulfate(1). It was felt that the hemoglobin was the direct or indirect cause of the renal vasoconstriction. This was indicated by the demonstration that intravenous infusion of purified commercial human hemoglobin and autogenous hemoglobin in humans caused a decreased GFR and ERPF(3). Also, examination of renal biopsy or autopsy specimens revealed the same histologic renal damage in dogs following intravenous infusion of homologous hemoglobin solutions made from either hemolyzed red blood cells or crystalline hemoglobin(4). However, these reports did not deal with the acute renal effects of homologous hemoglobin in dogs nor the effects of red blood cell stroma without hemoglobin or a protein free filtrate made from homologous red blood cells.

From these present experiments it is apparent that a protein free filtrate of the dogs' red blood cells does not alter GFR or ERPF. The hemoglobin free preparation of red blood cell stroma was also without effect during the active infusion periods. However, it is interesting to note the decrease in both GFR and ERPF in the first post-infusion period. A significant decrease in both GFR and ERPF was demonstrated during infusion of the partially purified hemoglobin but the decrease was not as dramatic as with the lysed red blood cell solutions, even though the quantity of hemoglobin administered was quite high in both infusion periods (153 and 145 mg/kg/min) and plasma hemoglobin levels were as high as noted previously(1,2). These findings indicate that hemoglobin accounts for most of the renal vasoconstriction during infusion of homologous lysed red blood cell solutions but also suggest that the red blood cell stroma probably has an additive effect. This correlates well with the observation in rabbits that sudden death resulted during or immediately after injection of homologous hemolysed blood whereas if stroma was removed by centrifugation much larger amounts of the solution were tolerated (5).

It was noted that initial passage of the lysed homologous blood cells through the liver by direct portal infusion was accompanied by an insignificant effect on GFR and ERPF(2). This was interpreted as due to either a delay of the solution in reaching the general circulation or modification of the vasoconstrictor substance. Basis for believing that the liver specifically altered the homologous hemoglobin might be enhanced if differences in renal response could be demonstrated by infusions of heterologous lysed red blood cells through both peripheral and portal veins.

These experiments demonstrate that peripheral infusion of heterologous lysed red blood cells obtained from the human, horse, cow and sheep caused a significant decrease in GFR and ERPF similar to the effects of homologous lysed red cells. Peripheral infusion of cow red cell lysate caused a decreased GFR and ERPF beyond the period of active infusion and was accompanied by tachycardia, tachypnea and a bleeding tendency. These signs and symptoms are quite similar to those seen in sensitized transfusion reactions in dogs or the reaction of dogs to massive transfusion of heterologous (human) whole blood(6).

All dogs which received the heterologous red cell lysates intraportally showed a significant fall in GFR and ERPF. In almost one half of these experiments a moderate decrease of peripheral arterial blood pressure was noted. The uniform fall in GFR and ERPF in these experiments is probably not due to the moderate vasodepressor effect since mean blood pressure remained above the critical level of 50-60 mm Hg below which the GFR and ERPF will start to fall(7). The interesting observations noted with the peripheral infusion of cows' lysed red cells did not occur during the portal infusion of this solution.

It is apparent that the 'protection' afforded the kidneys by passage of homologous lysed red cells through the liver is not present when the heterologous lysed cells are similarly infused. It would appear, therefore, that the 'protection' is not simply a mechanical slowing of the infusion's appearance in the general circulation. More likely a specific modification of the vasoconstrictor substance occurs in the liver when homologous hemoglobin is used. This mechanism is incapable of efficient modification of heterologous hemoglobin.

Summary. The renal vasoconstriction associated with peripheral intravenous infusion of homologous lysed red blood cells in dogs is primarily caused by hemoglobin although the red blood cell stroma appears to have an additive effect. Peripheral intravenous infusion of heterologous lysed red blood cells of human, cow, horse, and sheep origin cause a significant decrease in GFR and ERPF in dogs. Portal vein infusion of heterologous lysed red blood cells through the liver causes a significant decrease in GFR and ERPF in dogs and is frequently associated with moderate decrease in arterial blood pressure. These experiments suggest that the protection afforded the kidney by initial passage of homologous red cell lysates through the liver is due to specific modification of the hemoglobin, a process the liver is unable to perform with heterologous red cell lysates.

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Monoenoic Fatty Acids in Dogfish Livers: Isomers of the C_{16}, C_{18}, C_{20}, and C_{22} Series. (26868)

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Recently, interest has been expressed in the structures of unique monoenoic fatty acids from vegetable(1) and marine-animal oils(2) and from human fecal lipids(3). Dogfish (*Squalus acanthias*) liver oil is a rich source of many monoenoic fatty acids(4) whose positional isomers have not been reported. We have isolated most of these fatty acids from the liver oil and have determined their structures by oxidative degradation of the double bonds followed by analysis of the resulting fragments as methyl esters by gas-liquid chromatography (GLC). This paper reports the principal structures of the C_{16},

C_{18}, C_{20}, and C_{22} monoenoic fatty acids. Suggestions are given on the possible biogenetic origin of these isomers.

Experimental. The oil obtained from a large number of livers of dogfish caught off the coast of British Columbia was extracted by steaming and pressing(5). Methods that were applied previously to the fractionation of fatty acids from fish oils(6) were adapted to isolation of the monoenoic fatty acids. Fatty acids, free of nonsaponifiable constituents, were obtained by saponification of the oil and by extraction of the aqueous soap solution with ether prior to acidification. About 108 g of urea were placed in 270 ml of methanol, and the mixture was refluxed to effect solution. After addition of 30 g of fatty acids, the solution was mechanically stirred to promote formation of crystals and allowed to cool to 25°C. The mixture was held at this temperature for 1 hour. The filtrate was discarded, and the fatty acids from the crystals were recovered (A, 17.0 g). Overnight crystallization of A from 102 ml of acetone at –5°C yielded fatty acids from the filtrate (B, 11.4 g) that were found to be mostly monoenes (>90%) by GLC. GLC was carried out with a Research Specialties Instrument, model 600, equipped with a 10-ft. × ¼-in. column of 3% diethylene glycol succinate polymer (DEGS) on siliconized(7) Chromosorb W (80/100 mesh). The column was operated at 170°C and at a flow rate of 35 ml of argon per minute. A high-vacuum fractional distillation (750 mm spinning-band column, 0.6 mm Hg pressure at distillation head) of B after esterification with methyl alcohol and sulfuric acid yielded fractions of methyl esters that were mostly monoenes of one chain length. Since these fractions were not sufficiently pure for oxidation studies, they were fractionated further by preparative GLC in 0.5 ml amounts, and C_{18}, C_{20}, and C_{22} monoenes were isolated in high purity (98-100%). The C_{16} monoene contained a large amount of palmitate that does not interfere with oxidation studies. The preparative GLC* was carried out with a Beckman "GC-2 Gas-chromatograph" equipped with a 5-ft. × ½-in. column packed with 40% precipitated DEGS on Chromosorb (30/60 mesh)(8). The flow rate was 1300 ml of helium per minute. Infrared analysis of methyl esters from the whole oil and of each fraction obtained by GLC did not indicate the presence of *trans*isomers (10.3 μ).†

Oxidations of the monoenes were carried out by adapting the procedure described by Haverkamp-Begemann and co-workers(9). About 50 mg of methyl esters were oxidized with potassium permanganate at 25°C in 0.6 ml of glacial acetic acid. The potassium permanganate was added, a few crystals at a time, over a period of 5 hours. The reaction was conducted in a test tube that was mechanically agitated. Monocarboxylic acids and half-esters of dicarboxylic acids were isolated by the method reported recently by Fulco and Mead(10). The oxidation products were converted to methyl esters with diazomethane in ether(11) and were analyzed by GLC at column temperatures varying from 120° to 150°C, depending upon the chain lengths of the esters involved. Flow rates varied from 30 to 40 ml of argon per minute. Analyses were carried out with the Research Specialties Instrument equipped with a 6-ft. × ¼-in. column packed with 8% DEGS on Chromosorb W (80/100 mesh)(8). Pure mono- and di-carboxylic acid esters were used as standards in analysis of the fragments.

Results and discussion. The work of Haverkamp-Begemann and co-workers(9) indicated that the permanganate-acetic acid oxidation technic causes formation of small amounts of secondary oxidation products. Smith and co-workers(1), however, found this method preferable for determination of the structures of monoenoic fatty acids in seed oils. Infrared analyses indicated that double bonds in the monoenoic fatty acids are in the *cis*-form. It is recognized, however, that small amounts (<3%) of *trans*-

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† Infrared spectra were measured as films on sodium chloride plates using a Baird-Atomic spectrophotometer, model NKI.

TABLE I. Monoenoic Fatty Acids in *Squalus acanthias* Liver Oil.

Fatty acid	% of unsaturated fatty acids	% of total fatty acids
cis- 9-hexadecenoic acid	9.1	6.1
cis- 9-octadecenoic "	36.9	24.7
cis-11-octadecenoic "	14.6	9.8
cis- 9-eicosenoic "	6.0	4.0
cis-11-eicosenoic "	7.6	5.1
cis-11-docosenoic "	10.0	6.7
cis-13-docosenoic "	2.7	1.8

double bonds are not readily detectable by infrared analysis.

The procedure for isolation and identification of monoenoic fatty acids from dogfish-liver oil may also have application to the isolation of these fatty acids from other complex mixtures. Dogfish-liver oil is a rich source of C_{20} and C_{22} monoenoic fatty acids that are not found in significant amounts in most fish oils. Monoenoic fatty acids (Table I) were found to comprise 58% of total fatty acids and 87% of unsaturated fatty acids in dogfish-liver oil extracted from a substantial quantity of livers. The remainder of the unsaturated fatty acids are primarily C_{20} and C_{22} polyenes, and only small amounts of C_{20} and C_{22} dienoic and trienoic fatty acids were present in the oil. The monoenoic fatty acids reported here were found previously in various natural oils(12), and Hopkins and Chisholm(13) isolated 11% of 11-eicosenoic acid from liver oil of dogfish caught off the eastern coast of Canada. These authors also qualitatively identified 9-hexadecenoic acid in the same oil. The *cis*-11-octadecenoic acid has been found in animal and vegetable oils (12), but it is not known to what extent this acid is present in fish oils.

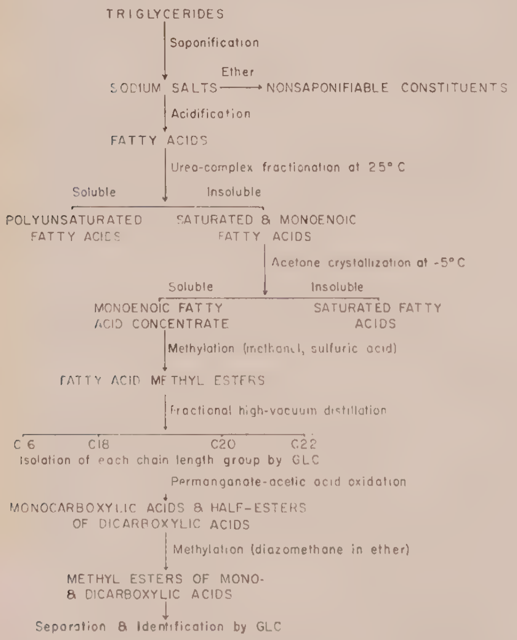
TRIGLYCERIDES
Saponification
Ether
SODIUM SALTS
NONSAPONIFIABLE CONSTITUENTS
Acidification
FATTY ACIDS
Urea-complex fractionation at 25° C
Soluble
Insoluble
POLYUNSATURATED FATTY ACIDS
SATURATED & MONOENOIC FATTY ACIDS
Acetone crystallization at -5° C
Soluble
Insoluble
MONOENOIC FATTY ACID CONCENTRATE
SATURATED FATTY ACIDS
Methylation (methanol, sulfuric acid)
FATTY ACID METHYL ESTERS
Fractional high-vacuum distillation
C16
C18
C20
C22
Isolation of each chain length group by GLC
Permanganate-acetic acid oxidation
MONOCARBOXYLIC ACIDS & HALF-ESTERS OF DICARBOXYLIC ACIDS
Methylation (diazomethane in ether)
METHYL ESTERS OF MONO- & DICARBOXYLIC ACIDS
Separation & Identification by GLC

FIG. 1. Scheme for isolation and identification of monoenoic fatty acids in *Squalus acanthias* liver oil.

Biogenetic origins of monoenoic fatty acids. Lovern viewed the biogenesis of fatty acids in dogfish-liver oil in terms of both a hydrogenation process(14) and a chain elongation process(15). The structural analyses reported here allow a further evaluation of these concepts. The position of the double bond in the isomers reported in Table I suggests that they are formed by chain elongation by addition of acetate units. If 9-octadecenoic acid is elongated by addition of acetate units, the $\Delta 9$ double bond of the C_{18} acid becomes $\Delta 11$ in the C_{20} series and $\Delta 13$ in the C_{22} series. The 11-octadecenoic acid may be formed by elongation of 9-hexadecenoic acid with acetate units, and 11-docosenoic acid may be synthesized, similarly, by elongation of 9-eicosenoic acid. These relationships may be represented thusly:

9-hexadecenoic→ 11-octadecenoic
9-octadecenoic → 11-eicosenoic ⟶ 13-docosenoic
9-eicosenoic ⟶ 11-docosenoic

Fatty acids having a $\Delta 9$ double bond thus appear to be the progenitors, by addition of acetate units, of most of the monoenoic fatty acids in dogfish-liver oil. Stoffel and Ahrens (6) suggested that C_{16} unsaturated fatty acids in menhaden oil may be formed from C_{18} fatty acids by loss of 2 terminal carbon atoms. The position of the double bond in the C_{16} monoenoic fatty acid from dogfish-liver oil suggests that it may have arisen similarly from oleic acid.

Summary. Structural isomers of monoenoic fatty acids in dogfish (*Squalus acanthias*) liver oil were determined. These fatty acids comprised 58% of total fatty acids and 87% of unsaturated fatty acids of the oil.

Although it is known that polyunsaturated C_{20} and C_{22} fatty acids are present in quantity in most fish oils, dogfish-liver oil was found to contain substantial amounts of C_{20} and C_{22} monoenoic fatty acids as well. The following isomers were found in the oil: *cis*-9-hexadecenoic acid (6.1%), *cis*-9-octadecenoic acid (24.7%), *cis*-11-octadecenoic acid (9.8%), *cis*-9-eicosenoic acid (4.0%), *cis*-11-eicosenoic acid (5.1%), *cis*-11-docosenoic acid (6.7%), and *cis*-13-docosenoic acid (1.8%). It is suggested that most of the monoenoic fatty acids of dogfish-liver oil may be formed by addition of acetate units to oleic acid and other fatty acids having a $\Delta 9$ double bond.

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Passive Cutaneous Anaphylaxis in Mice and Chickens. (26869)

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That the homograft reaction has many properties in common with delayed-type hypersensitivity is an established fact. Billingham *et al.*(1) demonstrated the passive transfer of the reactivity against skin homografts in guinea pigs is by cells and not by serum. The radiosensitivity of the homograft reaction(2,3) is of the same order of magnitude as that of the delayed hypersensitivity reaction(4,5), and much lower than that of soluble antibody formation(6). Nevertheless, antibodies directed against cell antigens often have been found in the serum of animals presensitized with homologous leukocytes(7) or lymphoidal cells(8,9), or bearing skin homografts(10,11). These circulating antibodies were detected by determining their cytotoxic activity *in vitro*(8,11,12) or by their ability to suppress antibody-forming activity of donor-type lymphoidal cells *in vivo* (9,13). Thus, it is apparent that the mechanism of rejection of homologous tissues is a complex one, and the relative role played by different factors is not well known; in order to understand this mechanism better, it seems necessary to use classical immunological methods along with *in vivo* assays for homograft reactions.

Recently, in other laboratories as well as in ours, transplantation antigens have been obtained in soluble form(14-16). Using these antigens, one can take a serological approach both to the question of the relative specificities of cells and serum and to identification of the antigens. We have used the passive cutaneous anaphylaxis (PCA) reaction with a well-known antigen-antibody system in the mouse as a preliminary step toward this approach. Our primary interest is in transplantation problems in mice; we included also some experiments of PCA in chickens because data are lacking on the subject, and because a great part of the embryological studies and many investigations on development of the immune machinery and on tolerance(17) have been conducted in those animals. The present study was limited to the following objectives: (a) to test the findings of Munoz and Anacker(18) on sensitivity of PCA in the mouse; (b) to determine whether the phylogenetic relationship between source of antibodies and the test animal influences the sensitivity of the test in mice (isologous *vs* homologous *vs* closely related heterologous *vs* distantly related heterologous), a trend indicated in the guinea pig by Ovary(19), (c) to develop a satisfactory reverse passive cutaneous anaphylaxis (RPCA) technic in the mouse, and (d) to develop a satisfactory PCA technic in the chicken.

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‡ Operated by Union Carbide Corp. for U. S. Atomic Energy Commission.

Materials and methods. 1. *Animals.* Twelve to 16-weeks-old C31F$_1$/Cum (C3H/Anf Cum ♀ × 101/Cum ♂) mice (H-2^{kk}), to be henceforth referred to as (C3H × 101)F$_1$, and LAF$_1$/J (C57L/J ♀ × A/HeJ ♂) mice (H-2^{ba}), to be henceforth referred to as LAF$_1$, were used. Their approximate weight was 25 g. Three-weeks-old white leghorn chicks weighing ~ 150 g were used.

2. *Antigens.* Crystalline bovine serum albumin (BSA) (Pentex Co.), was used in most experiments. Solutions of BSA were prepared the same day of experiment in 0.15 M NaCl in the case of RPCA, and in 0.15 M NaCl containing 0.5% Evans Blue dye in the case of PCA. Rat whole serum was used in one set of experiments.

3. *Antisera.* Anti-BSA sera were obtained from rabbits, chickens, rats (Sprague-Dawley), and mice (C3H × 101)F$_1$, and titrated quantitatively for their antibody nitrogen (AbN) content(20). The μg AbN/ml for the various sera was as follows: rabbit anti-BSA, 340; chicken anti-BSA, 588; rat anti-BSA, 134; mouse anti-BSA, 168. Chicken and mouse antisera against rat serum were produced respectively by repeated intravenous injection of whole rat serum in full-grown chicken, and by repeated intraperitoneal injection of rat serum in Freund's adjuvant in (C3H × 101)F$_1$ mice. Because of the complexity of the antigenic material, it was not possible to determine the AbN content of these antisera; their relative "strength" was estimated by the interfacial method (ring test) using the usual 4-fold dilution method. Then the titers of these antisera were adjusted to 4^{-5} by appropriate dilution with 0.15 M NaCl.

Two-fold serial dilutions of the antisera were always made on the same day of experiment. The diluent was 0.15 M NaCl, except in the RPCA experiments. In this case non-reactive Tyrode's solution was used as a diluent after we observed that in some cases 0.15 M NaCl controls showed weak non-specific reactions.

4. *PCA in mice.* PCA was induced according to the technic of Ovary(21) and Munoz and Anacker(18) with slight modifications. A very mild anesthesia was induced by leaving the animals in an atmosphere saturated with ether for 40 seconds. During the anesthesia the back was shaved gently with an electric clipper, a longitudinal line was drawn along the center of the back with a felt pen, and 0.05 ml of antiserum and 0.15 M NaCl control, or of 2 different dilutions of the antiserum were injected intradermally right and left of the central line using a 27-gauge needle. By the end of this operation the animals were almost completely awake. After 3 hours, 1.25 mg of BSA (0.025 mg/g body weight) in 0.25 ml of saline containing 0.5% Evans Blue was injected into the lateral tail vein. When rat serum was used as an antigen, the amount of protein injected was 2.5 mg. The mice were decapitated 30 minutes later and the inner surface of the skin was examined for dye accumulation. The reactions were scored from 1+ to 4+ according to their diameter, as reported by Ovary(21). The 2 negative controls were (a) 0.15 M NaCl instead of the antiserum,

and (b) a challenging saline injection containing dye without the specific antigen.

5. *PCA in chickens.* This test was conducted with the same time sequence as that in the mice. No anesthesia was necessary; 0.1 ml antiserum or 0.15 M NaCl control was injected intradermally in the lateral thoracic region. We found that upon delivery the fluid easily diffuses into the hypodermal tissues; to avoid this diffusion, the direction of the needle was reversed after penetrating the skin, and the antisera was injected in the superficial layers. The immediate appearance of a satisfactory injection was a whitish wheal, which disappeared in 10 minutes. The antigen was injected by the wing vein (10 mg in 2 ml of the standard BSA-dye solution).

6. *RPCA in mice.* This test was performed using the 2 technics of Ovary(19). According to the first technic, BSA was injected intravenously (2.5 mg/mouse or 0.1 mg/g of bodyweight). Twenty-four hours later, 0.05 ml of an appropriate dilution of the antiserum was injected intradermally as in the direct PCA, and 10 minutes later 0.25 ml of the standard dye solution was injected intravenously. The animals were killed 30 minutes later and the reactions evaluated. Our modification of this technic was an increased delay between injection of antiserum and the dye; we found that when the dye was injected simultaneously with the antiserum, a weak false positive reaction always appeared in the controls; the interval we used was the shortest that allowed us to have completely negative controls (approximately 10 minutes).

According to the second technic, the mice were inoculated with 0.002 mg antigen (BSA) intradermally, then injected intravenously with 0.25 ml of antibody dilutions plus 0.25 ml Evans Blue at the standard concentration. The reactions were read 30 minutes later.

Results and discussion. Experiment 1. PCA in mice with isologous vs *homologous antiserum.* The test was performed in (C3H × 101)F_1 and LAF_1 mice, and anti-BSA serum was obtained in (C3H × 101)F_1 mice. In both recipients the minimum amount of anti-BSA required to give a positive reaction was 0.007 μg AbN. Therefore, in this combination of strains there is no difference of sensitivity of PCA between isologous and homologous systems. The sensitivity of the reaction in mice confirms the findings of Munoz and Anacker(18) and under these conditions is comparable with that in guinea pigs(21).

Experiment 2. PCA in mice with antisera from different sources. The PCA test was performed in (C3H × 101)F_1 mice with mouse [(C3H × 101)F_1], rat, rabbit and chicken anti-BSA sera. The results (Table I) clearly demonstrate that the sensitivity of the test in mice with the 4 antisera used is dependent upon the phylogenetic relationship between source of antiserum and recipient; *i.e.*, mouse > rat > rabbit > chicken antiserum. Note that chicken antiserum failed to elicit a positive reaction even with

TABLE I. PCA and RPCA in Mice* (BSA-Anti-BSA Reaction).†

Dose of antibody (μg AbN per site) intradermally	Source of antiserum							
	Mouse		Rat		Rabbit		Chicken	
	PCA	RPCA	PCA	RPCA	PCA	RPCA	PCA	RPCA
2.0	++++	++++	++++	++++	++	+++	—	—
1.0	++++	++++	+++	++	++	++	—	—
.5	++++	+++	+++	++	+	+	—	—
.25	++++	++	++	+	+	—	—	—
.125	+++	++	+	+	—	—	—	—
.062	+++	+	+	—	—	—	—	—
.031	++	—	—	—	—	—	—	—
.015	+	—	—	—	—	—	—	—
.007	+	—	—	—	—	—	—	—
.003	—	—	—	—	—	—	—	—

* According to Ovary's "first technic"(19).

† Antigen dose per animal was 1.25 mg for PCA and 2.5 mg for RPCA.

2 μg of AbN. The results obtained with rabbit antiserum agree with those reported previously by Ovary(21) under comparable conditions.

Experiment 3. RPCA in mice with antisera from different sources. In the guinea pig, horse antisera cannot elicit a positive PCA reaction, but they can trigger a positive RPCA reaction, provided the test antigen is a globulin from a source closely related to the recipient (*e.g.*, rabbit gamma globulin) (19). Reasoning from this type of observation, Ovary deduced that the RPCA reaction is dependent upon the binding capacity of the antigen to the recipient tissues. If this is true, RPCA could be used to screen antigen-recipient affinities and may have an application in the study of transplantation antigens and of γ-globulin allotypes. It was of greater interest for us to know if and to what extent in the mouse RPCA might be dependent on "antibody-binding." Therefore, we determined RPCA according to Ovary's first technic with the same antisera that we had used in the PCA tests. We expected that the BSA antigen, being distantly related to the mouse, would not "bind" the recipient's tissues. This was confirmed by the negative reaction observed with chicken anti-BSA serum (Table I, last column). RPCA reactivity patterns were similar to those observed with PCA, except for a slight decrease in sensitivity (Table I). The delay between antiserum and dye injections could have been sufficient to permit "binding" of the injected antibodies to the tissues with a consequent reaction mechanism no different from that of the PCA. An experiment was performed to test this possibility using Ovary's "second technic" of RPCA(19), in which no delay between antiserum and dye exists. Again the same reactivity patterns were obtained (Table II). Thus, these results suggest that the antibodies can bind the recipient tissues during the reaction itself when they are of homologous or closely related heterologous origin. It can be concluded that, in order to have a reaction solely dependent on antigen-recipient relationship, only distantly related heterologous antisera should be used to perform RPCA in the mouse. To investigate the effect of an antigen more closely related to the mouse than BSA, whole rat serum was used as an "antigen," which we assumed would bind to some extent the recipient tissues, especially because of its globulin content. This reaction was also dependent upon the source of antiserum (Table III). The slightly positive reactions obtained with relatively high concentration of chicken antiserum in experimental animals are not significant since comparable results were obtained with the controls (column 3). It is likely that these false-positive reactions reflect a cross-reaction between chicken antibodies directed against rat serum proteins and mouse tissue antigens, for we have observed in Ouchterlony plates precipitin band formation between chicken anti-rat serum and mouse serum proteins.

TABLE II. RPCA* in Mice (BSA-Anti-BSA Reaction).†

Dose of antibody (μg AbN) intrav.	Source of antiserum	
	Mouse	Chicken
84	++++	—
21	++	—
5	++	—
1.25	+	—
.31	—	—
.07	—	—

* According to Ovary's "second technic"(19).
† Dose of antigen inj. per site: 2 μg.

TABLE III. RPCA* in Mice with Anti-Rat Sera.†

Antiserum dilution	Source of antiserum			
	Chicken	Mouse	Chicken	Mouse
	(Experimental)		(Control)‡	
1	++	++++	++	—
1:2	+	+++	+	—
1:4	±	+++	±	—
1:8	—	+++	—	—
1:16	—	+++	—	—
1:32	—	+++	—	—
1:64	—	++	—	—
1:128	—	+	—	—
1:256	—	—	—	—

* According to Ovary's "first technic"(19).
† Chicken and mouse antisera had the same interfacial titer (4^{-5}).
‡ No antigen.

Experiment 4. PCA in chickens with homologous and heterologous antisera. PCA can be demonstrated in chickens, although its sensitivity is considerably lower than that in

TABLE IV. PCA in Chickens (BSA, Anti-BSA Reaction).*

Dose of antibody (µg AbN/site)	Source of antiserum	
	Chicken	Mouse
10	++++	++
5	+++	++
2.5	+++	±
1.25	++	—
.62	++	—
.31	+	—
.15	—	—

* Antigen dose/animal = 50 mg (0.3 mg/g of body wt).

the mammals (Table IV). Again we found that in chickens sensitivity is higher with homologous than with distantly related heterologous antiserum against the same antigen. Although no reaction was elicited by chicken antiserum in the mouse (Table I, column 8), mouse antiserum was capable of inducing a positive though weak PCA reaction in chickens.

Summary and conclusions. The sensitivity of the PCA test with different anti-BSA sera was tested in mice. Sensitivity is greatest with isologous or homologous antisera; *i.e.*, as little as 7×10^{-3} µg AbN produced a positive reaction. In contrast, 62 and 250×10^{-3} µg AbN were necessary when rat and rabbit antisera were used, respectively; when chicken anti-BSA was used, the test was negative with as high as 2 µg AbN. The sensitivity of the PCA reaction in mice with isologous and homologous antiserum suggests that it could be a useful tool in screening for circulating antibodies against transplantation antigens. PCA in chickens is described for the first time. Although the sensitivity of this reaction relative to that in mammals is low, it can be elicited with a distantly related antiserum, such as mouse anti-BSA. RPCA in mice was found to depend on the antiserum-recipient relationship when either BSA or rat whole serum was used as antigen.

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Transmembrane Potentials of Cultured Mouse Dentinal Pulp Cells.* (26870)

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Odontoblasts are found in the pulp of teeth as a cellular layer adjacent to the dentin. The fine protoplasmic processes, Tome's Fibers, of these cells extend through the dentinal tubules to the dentino-enamel junction (D.E.J.) in the crown or the dentino-cemental junction (D.C.J.) in the root. Some workers have suggested that sensory function is mediated by the odontoblast(1,2); however, the prevailing opinion is that these cells are agents of dentin formation exclusively and that sensory function is mediated by nerve endings extending through the dentinal tubules to the D.E.J. or the D.C.J.(3,4).

Because of the lack of definitive information on this and other pulpal cell types, we have begun a study of the electrophysiological properties of these cells. Ultramicroelectrode technics for determining the transmembranal potentials of cells have yielded very instructive results on the changes in cellular activity and function of the cell membrane. This is a report of the measurement of membrane potentials of mouse dentinal papilla cells grown in tissue culture.

Methods. Pulpal cells obtained aseptically from newborn mouse molar tooth germs were cultured in plasma clots on narrow cover slips in Leighton tubes with a liquid nutrient consisting of Puck's N16(5) containing 30% calf serum. After 4 to 10 days of cultivation the cover slips were transferred to a microscope stage-chamber through which fresh culture medium could be perfused.

Ultramicroelectrodes, machine-pulled from 1 mm soft glass capillary tubing, were filled with 3 M KCl by boiling under reduced pressure and mounted on an inclined micromanipulator to allow access to the cells in the perfusion chamber. Impalement of single cells was observed under a binocular phase-contrast microscope. The electrodes were connected to a single ended D.C. amplifier, Grass P6 with a M.E.P. 6 probe, input impedance of 1000 megohms with input grid current adjusted to 10^{-12} amps, and oscilloscope recordings obtained with a Polaroid camera. The slides were fixed and stained for further morphological studies. Only electrodes with resistances of 10 to 40 megohms were used.

Results. Transmembranal potentials were measured in approximately 200 single cells in isolation or in clumps of cells from 20 cultures. On the basis of visual observations and electrophysiological data these cells can be classified into 3 groups. Table I is a summary of the morphological cell types and their membrane potentials recorded at 20 to 25°C. We have called the polygonal cell an odontoblast. Fig. 1, 2, and 3 show typical examples of the above cell types.

The potential measurements were made on cells viewed through a phase contrast microscope. As the micropipette penetrated the cell membrane, there was an abrupt negative deflection of the oscilloscope beam to a value which generally remained quite steady for a number of minutes. Just before penetration, dimpling of the cell wall could occasionally be observed, and this was marked by a slight shift, 3 to 5 millivolt, of either polarity. This latter observation is perhaps due to the mechano-transducer actions of micropipettes described by Brown and Wiesel(6). No spontaneous activity, either visual or electrical, has been observed in these cultured cells.

In general, tissue culture cells grown *in vitro* lose many of their morphological characteristics especially at the glass-clot interface where they tend to flatten and spread out. The area presented by the spread-out polygonal cells is about 7.5×10^{-5} cm². Taking into consideration the top and the bottom of the cell and assuming negligible

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† U.S.P.H.S. Senior Research Fellow.

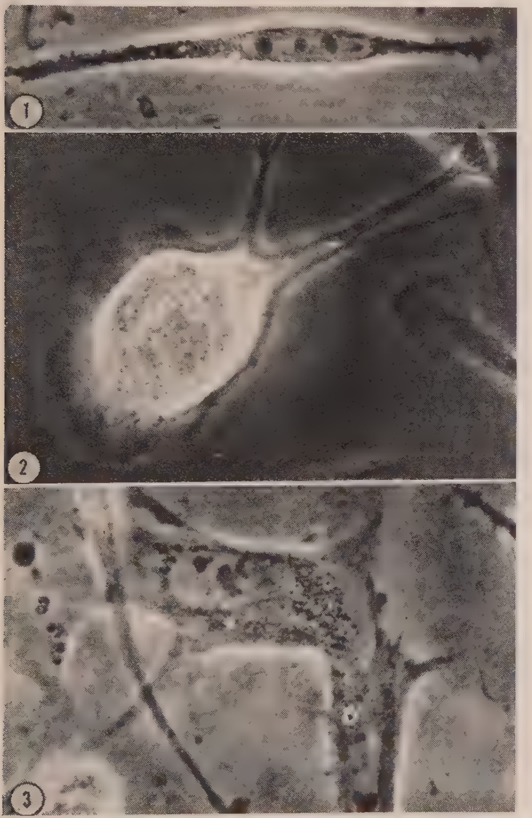
TABLE I. Cell Types and Membrane Potentials of Pulpal Cells Grown in Tissue Culture.

Cell type	No. of cells	Membrane potentials E_m = millivolts
1. Spindle-shaped cells, fibroblast-like	52	7.0 ± .6*
2. Round cells with or without a visible protoplasmic extension	37	19.3 ± 1.5
3. Polygonal cells with a long protoplasmic process	90	34.6 ± 1.4

* Stand. error of mean.

thickness one arrives at about 15×10^{-5} cm^2 as an estimate of total area of the cell.

Membrane resistances were determined on approximately 10 of the polygonal cells (odontoblasts) by the technic described by Sperelakis *et al.*(7) which consists of determining the increase in deflection height of a square-wave pulse when the electrode is intracellular. Membrane resistances of the range of 3 to 4 megohms were observed. No attempt was made to estimate possible errors in the determination *e.g.*, an artificial increase in resistance due to pressure on the electrode, etc.



Phase micrographs of living cells in a 14-day-old culture of molar tooth germ dental papilla from a one-day-old mouse.

FIG. 1. Spindle-shaped fibroblast-like cell. ×213.

FIG. 2. A round cell with protoplasmic processes. ×213.

FIG. 3. Polygonal cells with protoplasmic processes. ×213.

Discussion. It is not possible from these studies to determine the physiological role of these pulpal cells. The moderately high membrane potentials recorded for the polygonal cells, odontoblasts, indicate that they might be classified with such cells as smooth muscle(8) and glandular epithelium(9). The anatomic position of the odontoblast, the presence of cholinesterase in its membrane (1) and a moderately elevated membrane potential could support the idea of sensory mediation. It is equally possible, however, that these data support the much older view that the odontoblast acts in a secretory manner for governing the deposition of calcium salts in the dentin. Nylen and Scott(10) found extensive concentrations of rough surfaced endoplasmic reticulum in rat's odontoblasts. This suggests a secretory function but does not rule out a sensory function for these cells.

In confirmation of an observation by Crill *et al.*(11), it was noted that the membrane potentials of grouped cells were higher than those of any isolated cell. Whether this is the result of different electrical resistive values of a cell membrane in isolation compared with cell membranes in intimate contact is not known. Other properties of cells, for example cytochemical features, are known to differ in isolated cells as compared to their counterparts in clusters(12). Just what mechanism is involved remains to be determined. It is possible that the round cell is an odontoblast in the terminal stages. The rounding or "balling up" of a cell is generally taken as a sign of necrosis. These cells were not present in all cultures and when seen were usually found as solitary cells.

Preliminary studies on the *in vivo* pulps of young dogs and rats have indicated the presence of at least 2 of the cell types found in tissue culture; many scattered cells throughout the pulp having membrane potentials of 5 to 10 millivolts and a few cells located along the dentinal border with membrane potentials of 25 to 45 millivolts. Work is continuing along these lines.

Summary. It has been possible to grow in tissue culture viable cells from tooth germs of young mice. Three cell types have been determined based on morphological and electrophysiological characteristics. The mouse odontoblast grown in tissue culture possesses a membrane potential which is considerably greater than that of the fibroblast and is in the range reported for smooth muscle and glandular cells. At present it is not possible to state if the physiological role of the odontoblast is sensory, glandular, or both.

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Iodine-Induced Submandibular Sialadenitis in the Hamster. (26871)

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During the course of some studies on the pathogenesis of iodine-induced thyroiditis in the hamster(1), other tissues, particularly those which are known to concentrate this element, have been examined microscopically. Damage to the submandibular (submaxillary) glands has been found uniformly; in contrast, the parotid and sublingual glands, as well as the gastric mucosa, appear to be normal. This communication presents a description of the changes in affected glands together with comparative data on radioiodine uptake, which may help to explain the difference in reaction between the submandibular glands and other structures.

Materials and methods. Histological observations have been carried out on the salivary glands of approximately 100 normal male hamsters, each weighing about 125 g, which had received various quantities of iodide as the potassium or sodium salts for periods lasting several hours to 10 days. The iodide was administered intraperitoneally in distilled water. Animals were sacrificed at intervals from one hour after a single injection up to after 10 days of multiple doses, usually once daily. Submandibular, sublingual and parotid glands as well as glandular portions of the stomach, were studied after fixation with neutral formalin and staining with H and E, PAS, and alcian blue technics. Iodide dosages ranged from 1.0 to 200 mg, since it was found that smaller amounts were ineffective.

TABLE I. Relative Concentration of I^{131} in Tissues, Expressed as cpm/mg Wet Weight.

Time after I^{131}		Parotid gland	Sublingual gland	Submandibular gland	Stomach	Testis	Thyroid
1 hr		37.4	35.8	112.0	31.1	—	2900
2 "	1	46.3	49.8	263.0	43.3	11.1	3050
	2	44.0	41.7	165.0	—	19.1	5730
	3	62.3	90.5	620.0	—	37.0	4230
3 "	1	67.5	60.0	346.0	54.2	21.4	6060
	2	48.6	52.5	166.0	—	22.4	13100
	3	18.4	18.0	96.0	—	9.6	9450
4 "		15.8	15.7	30.6	—	—	2800

Normal animals were given 25 μc radioactive iodine, without carrier, intraperitoneally and sacrificed from 1 to 3 hours later. Samples of 3 salivary glands, thyroid, testis, and glandular portion of stomach were weighed on a torsion balance, digested in 3N NaOH and counted with a sodium iodide crystal scintillation detector.

Results. The normal submandibular gland (Fig. 1) of the hamster is composed of acini whose serous cells contain large numbers of small basophilic granules which stain by the PAS or alcian blue technics. Small, most proximal, intralobular ducts are lined by columnar cells with basal nuclei and pale cytoplasm, with faintly PAS staining granules. The cells lining the distal ducts are smaller with eosinophilic cytoplasm. The cells of the collecting ducts are more squamous in appearance. The following description of glands from treated animals is a composite of observations made after doses of iodide of various sizes. Obviously changes develop more rapidly after large than small amounts of iodide.

Following a large dose, *i.e.*, 10 mg of iodide or more, changes are seen as early as 2 to 4 hours. The lobules are spread apart by swelling of the loose interlobular connective tissue. Inflammatory cells, both polymorphonuclear as well as mononuclear types, are found in these areas. The edema and inflammatory cells are related to distinct changes in the distal portions of ducts. The walls of these structures are infiltrated with polymorphonuclear cells; the lining epithelial cells may be completely necrotic. In addition, the duct lumina are filled with leukocytes, basophilic (PAS positive) material, and desquamated cells. In these early stages, too, large numbers of leukocytes are found in and about the walls of blood vessels. No hemorrhage is seen.

By 24 hours the alterations in the ducts are more severe. The lumina of the more proximal ducts are dilated but their lining cells do not appear damaged, save that they may be smaller. Leukocytes are found between the acinar elements, which even by this time have become atrophic and have begun to lose their basophilic (PAS and alcian blue positive) contents. With increasing time, regeneration of the duct epithelium begins even though administration of iodide continues; the lining elements have a squamous appearance, more like the cells of the collecting ducts than the normal distal duct cells. The duct system is conspicuously dilated.

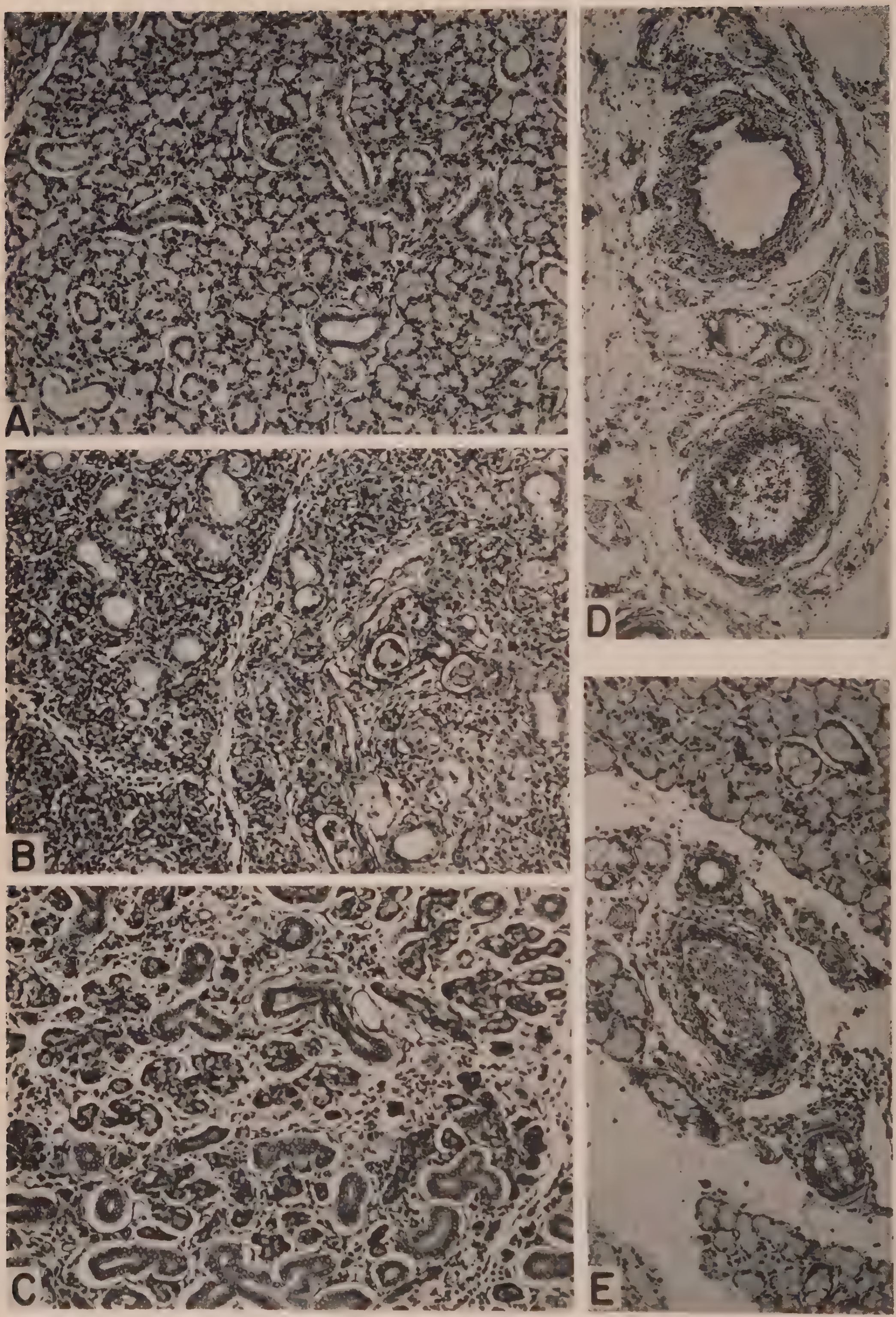
These alterations are usually diffuse. Sometimes, however, especially with lower dosage of iodine, single lobules will be involved, while neighboring ones remain normal in appearance.

If the process is followed into a more subacute phase, the regeneration of duct epithelium elements becomes more conspicuous, while the acinar elements exhibit atrophy.

No difference was found between sodium or potassium salts of iodide.

Studies of the sublingual and parotid glands as well as the glandular portions of the stomach failed to reveal any lesions.

Studies of radioactive iodine uptake of 3 salivary glands, glandular portion of the stomach, thyroid, and testis are presented in Table I. It will be noted that the concentration of iodide in the submandibular gland


A
B
C
D
E

is 3 to 5 times that of the other salivary glands in the first few hours after administration. Concentrations in thyroid and testis are high and low respectively as expected.

Discussion. Since the 1830's when iodides came to be used extensively in treatment of syphilis, clinicians have known that these compounds affect the salivary glands. Their presence in saliva(2) and the fact that painful swelling of the parotid glands ("iodine mumps") might develop when excessive amounts of potassium iodide are administered are well known(3,4). That a true sialadenitis may result from administration of large amounts of iodine to hamsters is of great interest with respect to these physiological and clinical experiences.

The distal portions of the submandibular duct system appear to suffer initial damage when iodide is administered in sufficient amounts. The other alterations described above are interpreted as secondary to the changes in the ducts. This site of injury is of great interest in view of the autoradiographic studies of Cohen *et al.*(5) who have demonstrated that radioactive iodide is localized to the proximal ducts of the submandibular glands of the hamster and is not found in the acinar portions or in the epithelial elements of the distal and collecting ducts. To explain this difference in localization between proximal portions, where presumably uptake and secretion occur, and distal portions, where injury is found, one may presume that the proximal duct cells remove the iodide from the blood and are not damaged in so doing, but that the concentration of iodide is high enough in the saliva further on to damage the lining cells of the distal portions of the ducts.

It is of interest to recall that in the therapy of syphilis with iodides patients might receive 10 to 30 g of potassium iodide each day(4). A dose of 12 g is equivalent to about 9 g of iodide, or 120 mg per kilo for a 75 kg individual. Such a dose is comparable to 10 mg for a 125 g hamster, an amount of iodide which produces marked changes in the submandibular gland.

The comparative studies of uptake of I^{131} by the 3 salivary glands of the hamster, reported above, confirm those of Myant and coworkers(5,6,7). The relative activity of the submandibular glands with respect to the others, which was found by these workers, is of the same order of magnitude, *i.e.,* about 5 times that for the parotids, as was observed here. The concentrating activity of the gastric mucosa is of the same order as that of the parotid and sublingual glands. Autoradiographic studies had previously demonstrated the uptake of iodine by glandular gastric mucosa of the hamster(8). That the submandibular glands, in contrast to the parotid and sublingual glands and glandular gastric mucosa, are the only structures involved when iodide is administered would appear to warrant the hypothesis that the concentrating ability and damage are causally related. That a certain dosage level must be reached before the alterations will appear further strengthens this hypothesis, one which could, of course, be further enhanced by studies in other species. In the rat, which does not concentrate iodine to any degree, no inflammatory change has been produced by excess iodine (unpublished observations). The species differences already reported by Cohen and Myant(7) provide data which may guide such studies.

Naturally, the production of damage in salivary gland which can best concentrate iodine is of great interest with respect to the iodine-induced thyroiditis studied in this laboratory(1). That a critical amount of iodide must be administered to produce this change in the thyroid(9) and that a high concentration of iodine must be reached in the gland if the change is to occur (unpublished observations) make most plausible the hypothesis that the inflammation is the response of a direct toxic effect of iodide on

FIG. 1. *A.* Submandibular salivary gland, normal hamster (H-532) (× 125). *B.* Submandibular gland (H-516) 3 days after administration of 20 mg iodide (× 125). *C.* Submandibular gland (H-528) 10 days after 200 mg iodide (× 125). *D.* Submandibular ducts (H-578) 4 hr after 200 mg iodide showing inflammatory reaction about and in walls of ducts (× 80). *E.* Submandibular duct showing necrosis wall and periductal inflammation 6 hr after 200 mg iodide (× 80).

the thyroid epithelial cells. Whether this hypothesis can be established with certainty remains for continuing studies to disclose.

The occurrence of spontaneous sialadenitis has been described in various species(10,11). The hamster is no exception(12). Salivary gland disease in this species is characterized by prominent intranuclear inclusions. These have not been observed in the salivary glands of any animals studied in this laboratory. Furthermore, no inflammatory changes have been observed in a large number of animals which had not received iodide. The possibility must, of course, be considered that iodide may activate a latent viral agent. If so, and such seems most unlikely, this agent is unaccompanied by inclusions and must be activated with great rapidity, *i.e.*, within 4 hours.

The sexual dimorphism of the submaxillary glands of the rat and mouse is well recognized(10). All of the observations reported herein have been in males. Whether such a change occurs in females remains to be studied. So, too, the effects of prolonged administration of iodide as well as the possible production of chronic sialadenitis are worthy of further investigation.

Summary. Administration of large amounts of potassium iodide to the hamster leads to an inflammatory reaction in the submandibular glands, but not in the parotid or sublingual structures. The primary site of damage is the distal duct system. Such selective damage by this agent may be related to the greater concentrating ability of iodine by the submandibular glands in contrast to the parotids and sublinguals.

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Establishment of Resistance to Gardner Lymphosarcoma (6C 3HED) and L#2 Lymphoma in C3H and A/He Mice.* (26872)

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The production of resistance to growth of Ehrlich carcinoma in C57 BL mice(1,2) and in Swiss mice(3) by intraperitoneal injections of X-irradiated Ehrlich carcinoma cells has been reported. Furthermore, Revesz(4,5) has found that if X-irradiated Ehrlich cells are mixed with non-irradiated cells in appropriate quantities there is inhibition of cancer cell growth and immunity is produced in the injected mice. However, if too few X-irradiated cells are present in the mixture there may even be an enhanced growth without immunity being produced.

We are reporting here the production of complete resistance in C3H mice to Gardner lymphosarcoma (6C 3HED) and L#2 lymphoma, and in A/He mice to Gardner tumor.

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Also partial resistance was produced in A/He mice to L#2 lymphoma. These immunized mice showed little or no protection when injected with a second completely viable non-irradiated tumor.

Materials and methods. The 113 C3H mice and 107 A/He mice used in these experiments were obtained from the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine, and from the Cancer Research Genetics Laboratory, Univ. of California, Berkeley. Both males and females, 7-11 weeks of age at start of experiments, were used. The mice were housed in groups of 5 in hanging metal cages, 5″ × 5″ × 7″, with wire bottoms, and were given water and Ralston Purina Laboratory Chow *ad libitum.*

The ascitic forms of the Gardner lymphosarcoma, Cancer Research Genetics Laboratory, and the L#2 lymphoma, obtained from Dr. Theodore S. Hauschka, Roswell Park Memorial Inst., Buffalo, N. Y., were employed. The Gardner tumor was harvested after 7-9 days' growth in C3H mice, in which tumor was maintained by weekly intraperitoneal injections of 0.2 ml of undiluted tumor. The L#2 tumor was obtained after 7-10 days' growth in A/He mice in which tumor was maintained by weekly intraperitoneal injections of 0.2 ml of tumor diluted 1:100 with 55 mM phosphate Locke's solution. Ascites tumors were withdrawn from the peritoneal cavity under sterile conditions, and employed untreated or diluted with phosphate Locke's solution. In the irradiation process 1-6 ml of undiluted tumor were placed in special sterile 50 ml balloon flasks which were rotated under the X-ray unit at a rate of 25-30 rpm at a temperature of 28-30°C. Tumor was X-irradiated with 2000 r, at a rate of 53 r per minute and a focal distance of 45 cm. Dose rate was calibrated in air for the 250 kv X-ray head having 0.21 mm of Cu, inherent. Additional filters were 1 mm of aluminum and 0.5 mm of Cu, parabolic. The half value layer was 2.1 mm of Cu.

All tumor injections were made intraperitoneally under sterile conditions. The Gardner tumor, containing about 240,000 cells per mm^3, was injected in amounts ranging from 0.2 ml diluted 1:100 with 55 mM phosphate Locke's solution to 0.2 ml undiluted. The L#2 tumor, containing about 150,000 cells per mm^3, was injected in amounts varying from 0.1 ml diluted 1:100 with phosphate Locke's solution to 0.5 ml undiluted. The quantities of each tumor used for the various experiments are shown in Table I.

Primarily this study involved 4 phases, attempts to establish resistance to Gardner lymphosarcoma in C3H and A/He mice, and to L#2 lymphoma in C3H and A/He mice. In each phase were 2 groups of mice, controls and test animals. Test animals initially received 8 injections (2 per week) of X-irradiated tumor. Four to 7 days after the last injection of X-irradiated tumor the mice received a challenging injection of non-irradiated viable tumor. A second challenging injection of viable tumor followed 20-90 days later (Table I). Control animals were untreated mice that received a single injection of non-irradiated tumor. Controls were utilized for the initial injection of X-irradiated tumor and for each of 2 challenges with non-irradiated tumor.

An additional phase of the study, not shown in the table, was to determine whether mice that were made immune to one of the tumors showed resistance to the second tumor. Consequently, after establishing immunity in C3H mice to Gardner lymphosarcoma and giving them 2 challenges with viable Gardner cells, they were then challenged 38 days later with 0.5 ml non-irradiated L#2 lymphoma cells. Similarly, C3H mice that were immunized and twice challenged with L#2 lymphoma cells, were subsequently challenged 30 and 80 days later with 0.2 ml (1:100 diluted) non-irradiated Gardner lymphosarcoma cells. Also, A/He mice that were immunized and twice challenged with Gardner lymphosarcoma cells, were subsequently challenged 40 days later with 0.1 ml (1:100 diluted) non-irradiated L#2 cells.

Results. The data in the table show that 8 injections of X-irradiated Gardner lymphosarcoma offered complete protection against growth of viable non-irradiated Gardner tumor in both C3H and A/He mice for both challenges. All of the C3H control animals

TABLE I. Production of Resistance to Gardner Lymphosarcoma (6C 3HED) and L#2 Lymphoma in C3H and A/He Mice.

Treatment*	Survivals‖	Survival time (days)	Amt and dilution of tumor
A. Gardner lymphosarcoma in C3H mice			
Controls	0/39	10–13 (12)§	.2 ml 1:100
Test animals—X-irradiated tumor	36/36	—	*Idem*
1st challenge (5)†	36/36	—	”
2nd challenge (22)‡	36/36	—	”
B. Gardner lymphosarcoma in A/He mice			
Controls	4/23	9–20 (12)§	.2 ml undil.
Test animals—X-irradiated tumor	23/23	—	*Idem*
1st challenge (4)†	23/23	—	”
2nd challenge (33)‡	23/23	—	”
C. L#2 lymphoma in C3H mice			
Controls	3/23	7–11 (8)§	.5 ml undil.
Test animals—X-irradiated tumor	15/15	—	.4 ml ”
1st challenge (5)†	15/15	—	.5 ml ”
2nd challenge (30)‡	13/15	(5)§	.5 ml ”
D. L#2 lymphoma in A/He mice			
Controls	0/30	9–14 (11)§	.1 ml 1:100
Test animals—X-irradiated tumor	24/31	18–30 (25)§	” 1:100–1:10
1st challenge (7)†	13/24	2–34 (21)§	” 1:100
2nd challenge (90)‡	6/13	13–33 (26)§	” 1:100

* Controls received 1 inj. of non-irrad. tumor. Test mice received 8 inj. of 2000 r X-irrad. tumor followed by 2 inj. of viable non-irrad. tumor (challenges).

† Days since 8th inj. of X-irrad. tumor.

‡ Days since 1st challenge.

§ No. in parentheses is avg.

‖ Ratios express No. of mice surviving, over total No. of mice in the group treated.

died and all except 4 of the A/He controls died. Similarly, 8 immunizing injections into C3H mice with X-irradiated L#2 lymphoma gave complete protection for the first challenge, while all except 3 controls died. All except 2 of these mice survived a second challenge.

In experiments with L#2 lymphoma and A/He mice 7 animals died as a result of injecting 2000 r X-irradiated tumor. A plausible explanation for these deaths is that there may have been inadequate X-irradiation thus allowing completely viable cells to remain. Average survival time of these 7 mice was 25 days compared to 11 days for the controls. Of the 24 remaining mice 13 survived the first challenge of non-irradiated L#2 tumor with an average survival time of 21 days, and 6 of these 13 survived a second challenge of L#2 tumor with an average survival time of 26 days. Survival time of the A/He test mice was significantly longer than that of the controls ($P<0.01$).

In preliminary attempts to determine whether mice that were immunized to one of the tumors were also immune to the second tumor, cross challenge experiments were performed. With C3H mice immunized to Gardner lymphosarcoma a challenge with L#2 tumor produced death in all except 3 of the 30 mice with no prolongation of survival time. C3H mice immunized with L#2 lymphoma and challenged 2 times with Gardner tumor produced death in all except 4 of 13 mice with an insignificant prolongation of survival time ($P>0.1$). A/He mice immunized with Gardner lymphosarcoma and challenged with L#2 tumor produced death in 8 of 10 animals but with a prolonged survival time ($P = .02$).

Discussion. In addition to employing X-irradiated cancer cells for production of immunity in mice a number of other technics and materials have been employed with varying degrees of success(1). However, more nearly complete protection has been afforded by use of X-irradiated cells. Different amounts and rates of X-irradiation were not studied in these experiments so that optimum irradiating conditions for the cancer cells may not have been achieved. A 2000 r dose, found to be most effective in our earlier investigation(1,2) with Ehrlich ascites carcinoma cells, was employed. It would appear that metabolizing and perhaps partially viable cancer cells are required for production of resistance since our earlier experiments showed that 2000 r to 4000 r treated Ehrlich ascites cells injected into C57 BL mice gave nearly an optimum production of resistance, while 8000 r was ineffective. Also Donaldson and North(6) have shown that a certain amount of nitrogen mustard is highly effective, although larger quantities reduce the effect.

To date we have not determined the mechanism involved in production of immunity by repeated injections of X-irradiated cells; however, in C57 BL mice immunized with X-irradiated Ehrlich carcinoma (1,2) blood serum, spleen, lung and liver tissues have high titres of complement-fixing antibodies. Such studies have not been made on tissues from animals immunized against Gardner lymphosarcoma and L#2 lymphoma. It is possible that X-irradiation alters the histocompatibility genes and this in turn produces different antigens, which results in an alteration of the array of antibodies in the animals. Another possibility is that X-irradiation modifies the cells in such a fashion as to alter and slow their growth and make them more antigenic.

It is not surprising perhaps that cross challenging of immunized mice gives different results with the various tumor-mouse combinations but it is most interesting that there is only slight protection. In this regard unpublished data in our laboratory show that C57 BL mice repeatedly injected with X-irradiated normal liver and spleen have no protection against growth of Ehrlich carcinoma cells.

Summary. It has been possible to produce complete protection in C3H mice to Gardner lymphosarcoma and to L#2 lymphoma, and in A/He mice to Gardner lymphosarcoma. Partial immunity was produced in A/He mice to L#2 lymphoma. This was accomplished by semiweekly intraperitoneal injections of 8 doses of 2000 r X-irradiated tumor. C3H mice immunized with Gardner and cross challenged with L#2 tumor and C3H mice immunized with L#2 tumor and challenged with Gardner tumor showed no significant amount of protection. However, A/He mice immunized with Gardner and challenged with L#2 tumor were partially protected. Plausible mechanisms of immunity production are discussed.

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Uptake of P^{32} by Pigeon Crop-Sac as Index of Lactogenic Hormone.* (26873)

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It has long been known that lactogenic hormone will produce a proliferative hypertrophy in crop-sac of pigeons with concomitant production of crop-milk(1). It has been shown that resultant growth and secretory processes appear to be associated with increased synthesis of nucleic acids and other phosphorous containing compounds(2,3). More specifically it has been demonstrated that crop-sac proliferation induced by systemic injection of lactogenic hormone rapidly incorporates P^{31} and P^{32}(4).

Recently improvements have been made in method of measurement of diametric response in pigeon crop-sac produced by intradermal injection of lactogenic hormone(5). Development of an improved, more sensitive and objective technic for bioassay of lactogen would be facilitated by correlation of this index of lactogenic activity in pigeons with technics involving incorporation of P^{32} into crop-sac.

Materials and methods. In first experiment, 2.8 μg lactogenic hormone in .1 ml distilled H_2O was administered once daily over one side of crop-sac for 4 days to common pigeons. Twenty-four hours after last injection, a solution of inorganic P^{32} having an activity of 100 μc per ml was administered intraperitoneally in dose of 10 μc per bird. Pigeons were sacrificed and weighed 4 hours after P^{32} injection and blood was collected. Crop-sacs were removed, dissected free of fat, cut approximately in half, and weighed on an analytical balance.

Crop-sac halves were prepared for P^{31} and P^{32} determination by digestion with nitric and perchloric acids and diluted to a volume of 100 ml. An aliquot was taken for P^{31} determination by photometric method, employing vanadomolybdate as color developing reagent. A 10 ml aliquot of undiluted acid digest was mixed with 10 ml scintillation solution containing POPOP, PPO and dioxane and placed in a Packard tricarb spectrometer for determination of P^{32}. Counting period was 30 to 50 minutes depending upon radioactivity of the samples. Conventional corrections were made for background and decay.

In the second experiment, 6 groups of rat pituitaries obtained from groups of animals given various treatments were macerated and diluted to 10 ml. Injections of .1 ml were injected intradermally over one side of crop-sac of pigeon daily for 4 days. Twenty-four hours after last injection of pituitary homogenate, 10 μc inorganic P^{32} solution were injected intraperitoneally. Pigeons were sacrificed and weighed 4 hours after P^{32} injection, crop-sacs removed, halved, and weighed on analytical balance. Diametric responses were estimated using standard calibrated disks for comparison(5) and P^{32} determined.

Results. In first experiment (Table I) specific activity (counts per minute of P^{32} per mg P) was increased in treated crop-sac halves as compared to control halves or blood. Total P per g tissue was significantly higher in crop-sac halves treated with lactogen than in control halves ($P<.05$), although lower than in blood ($P<.02$). P^{32} concentration (counts per minute per g tissue) was significantly higher in treated crop-sac halves than in control halves ($P<.01$).

In second experiment, lactogen equivalents (total amount injected per bird over 4 days) were calculated on basis of diametric response comparison to standard lactogen curve(6) (Table II). Comparisons of individual crop-sac weights and body weights *vs* per cent P^{32} uptake yielded correlation coefficients of .248

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TABLE I. Effect of Lactogenic Hormone on P^{31} and P^{32} in Crop-Sac and Blood.

	No. of birds	Total P/mg	Wt of crop-sac (g)	Counts/min. /mg P^{31}	mg P/g tissue	Counts /min./g tissue
1. Crop-sac, control	10	1.389 ±.148	1.287 ±.218	1353 ± 147	1.210 ±.113	1662 ± 282
2. Crop-sac, injected	10	2.008 ±.111	1.501 ±.132	1628 ± 168	1.418 ±.104	2324 ± 321
3. Blood	10	2.854 ±.898	1.446 ±.366	1017 ± 108	1.809 ±.114	1772 ± 587
		Significances*			1-2, P <.05 1-3, P <.01 2-3, P <.02	1-2, P <.01 1-3, not sig. 2-3, " "

* Computed by paired comparison "t" test.

TABLE II. Relationship of P^{32} Uptake to Diametric Response in Pigeon Crop Gland after Intradermal Injection of Rat Pituitaries.

Group	No. of birds	Wt of bird (g)	Wt of crop-sac	Diametric response (cm)	Lactogen equivalent (μg)	% P^{32} uptake × 10^4	Adjusted % P^{32} uptake × 10^4
1	14	328 ± 6	1.314 ±.092	2.89 ±.10	3.57	3.974 ± .314	4.57 ±.55
2	14	331 ± 6	1.672 ±.094	2.66 ±.16	2.69	4.528 ± .388	6.77 ±.08
3	16	306 ±12	.929 ±.044	3.09 ±.11	4.59	12.128 ±3.233	7.56 ±.79
4	13	303 ±14	1.040 ±.035	3.75 ±.13	10.32	10.165 ± .612	8.27 ±.91
5	16	314 ± 8	1.038 ±.060	3.09 ±.11	4.59	7.138 ± .426	6.34 ±.57
6	16	320 ± 9	1.260 ±.060	3.87 ±.11	12.05	9.179 ± .922	9.47 ±.99

and − .082 respectively. After adjustment of per cent uptake for these two factors(7), means of adjusted % P^{32} uptake were correlated with mean diametric response with a resultant coefficient correlation of .79.

Discussion. In first experiment, it would appear that P^{32} in blood had passed its specific activity peak, and hence had a faster equilibration rate than in crop-sac halves. Since specific activity is higher in injected crop-sac halves than in control halves, it would appear also that rate of incorporation of P^{32} at this time interval is considerably enhanced by local intradermal pretreatment with small amount of lactogenic hormone.

Previous study(4) has shown that P^{32} is more rapidly incorporated into tissue products of crop-sac after larger systemic doses of lactogenic hormone were administered. Present first experiment indicates that P^{32} incorporation into crop-sac half may be markedly influenced by local intradermal injection of small quantity of lactogenic hormone, and indicates possibility of use of this incorporation as index of lactogenic activity.

Diametric response has been used commonly for sensitive determination of lactogenic activity in crude and purified anterior pituitary extracts(5). Since mean diametric responses were shown to be highly correlated with % P^{32} uptake adjusted for crop and body weights, it would appear that such uptake is primarily dependent on lactogenic activity of crude pituitary homogenate injected. From standpoint of lactogen assay, adjusted P^{32} uptake as index of lactogenic activity not only retains sensitivity of diametric response technic, but adds element of objectivity.

Summary. Studies were conducted on ability of purified and crude lactogenic preparations to stimulate incorporation of P^{31} and P^{32} in crop-sac of pigeon. Lactogenic hormone injected intradermally over one side of crop-sac stimulated significant P^{31} and P^{32} incorporation as compared to control sides. Diametric responses were correlated with % P^{32} uptake after injection of crude pituitary homogenates with a resultant correlation coefficient of .79, indicating that P^{32} incorporation is primarily a function of lactogenic activity of homogenate injected.

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In vivo Transfer of a Thrombopoietic Factor.* (26874)

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The fact that the platelet count remains remarkably constant for months(1) although survival time of individual platelets is less than 2 weeks(2,3,4,5,6) suggests that there is a regulatory system or thrombocytostatic mechanism. Regulation implies feedback control in which unbalance acts as a stimulus to restore balance. According to this hypothesis, thrombocytopenia induced in an animal should stimulate thrombopoietic[†] activity to compensate for decreased platelet concentrations in the circulation.

Craddock *et al.*(7) and Matter and associates(8) reported increased thrombopoietic activity in dogs and rats, respectively, 4 days after production of severe thrombocytopenia by thrombopheresis. Both groups assumed that the thrombocytopenia was the sole stimulus for the thrombopoietic activity. However, recent unpublished experiments of the author showed that relatively moderate thrombocytopenia induced in rabbits by thrombopheresis resulted in pronounced thrombocytosis. Control experiments involving anesthesia and sham operations also produced very marked increases in platelet counts 3 to 4 days later. Because results were variable, the contribution of thrombocytopenia alone as a thrombopoietic stimulus was not amenable to quantitative analysis when thrombocytopenia was produced in rabbits by thrombopheresis.

These results indicated the importance of minimizing stress and suggested that a thrombopoietic factor in blood plasma might be demonstrable by intravenous transfer of plasma from thrombocytopenic donors to unstressed recipients. Provided that transfer of normal plasma was ineffective, a rise in platelet count could be interpreted as a response to a thrombopoietic factor induced by the thrombocytopenia.

Methods. Female albino rabbits (Blue Spruce) were used. Platelet counts were taken by puncture of an ear vein, using the direct method of Brecher and Cronkite(9). Although duplicate counts were not taken routinely, critical changes were often verified by a second technician. *A. Daily variations in circulating platelets.* Platelet counts of 9 rabbits were taken for 4 consecutive days and again on the seventh day. Three of the rabbits had been splenectomized more than a month prior to tests, but platelet levels had returned to pre-operative values within a week. *B. Transfer of plasma from normal donors to normal recipients.* Plasma was obtained from rabbits which showed steady platelet levels for at least one week prior to sacrifice. Blood was collected by heart puncture, performed under Nembutal anesthesia. Each 7.5 ml was put in a Becton and Dickson 3200 KA tube which provides a large excess of heparin. Within 2 hours of bleeding, 25 ml plasma were injected intravenously into each of 15 unanesthetized recipients, 4 of

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[†] It is recognized that the proper term is thrombocytopoietic but for convenience it is contracted to thrombopoietic.

TABLE I. Production of Thrombocytopenia in Rabbits and Effect of Injecting Their Plasma on Platelet Count of Normal Recipients.

Donor			Recipient			
				Platelet count, $10^3/mm^3$		
Treatment	Interval until bled	Platelet count when bled, $10^3/mm^3$	Dose, ml/kg	Day 0	Day 4	Increase, %
Myleran, 48 mg/kg	12 days	90	8.1	500	690	138
			7.8	375	460	123
Myleran, 26 mg/kg	43 "	165	8.1	325	710	218
			8.1	405	640	157
APS	20 hr	125	6.8	475	740	156
"	20 "	175	6.6	390	760	195
"	2 "	215	6.0	385	1,035	269
"	3 "	10	1.9*	445	580	131
"	3 "	—	6.6	340	710	209
			5.2	415	765	184
			2.7*	605	1,015	167
"	4 "	—	6.6	720	735	102
"	4½ "	160	6.0	480	860	179
"	3 "	—	7.4*	500	630	126
			8.1*	705	940	133
Mean ± S.D.				471 ± 121		166 ± 44

* Splenectomized recipients.

Brackets indicate that donor plasma was pooled or that several recipients received same donor plasma.

which had been splenectomized previously and all of which had demonstrated normal and constant control platelet levels for at least one week prior to injection. Doses varied from 5.9 to 8.1 ml donor plasma per kg recipient body weight. *C. Transfer of plasma from thrombocytopenic donors to normal recipients.* Two animals were made thrombocytopenic by administration of 1.4-dimethane sulphonyloxybutane (Myleran, Burroughs Wellcome Co.), and 8 were given anti-platelet serum (Table I). Myleran was homogenized with peanut oil in a concentration of 100 mg/ml and administered subcutaneously. Anti-rabbit platelet serum was prepared in dogs by a technic described by Elliott and Whipple(10), with slight alteration of antigen injection schedule. It was not adsorbed with erythrocytes or leukocytes. After intravenous injection of 5-8 ml anti-platelet serum into rabbits, the platelet counts fell to a few thousand per cu mm within a few minutes. The animals were bled between 2 and 24 hours later when platelet counts ranged from 10,000 to 215,000 per cu mm. Plasma was prepared and 25 ml injected in the same manner as normal plasma into 11 normal and 2 splenectomized recipients. Two additional splenectomized recipients received 10 ml.

Results. A. *Daily variations in circulating platelet count* (Curve A, Fig. 1). With rare exceptions, the platelet counts of healthy rabbits which had been in the laboratory for more than 10 days were between 350,000 and 700,000/cu mm and were quite constant. For the 9 animals subjected to consecutive counts, average platelet count was 514,000 ± S.D. 75,000/cu mm on day zero. On subsequent days, the group average platelet count was within 5% of this initial average, and maximum standard deviation for any day was 18%. Data for days 4, 5 and 6 of curve B, Fig. 1, were obtained by respectively comparing the counts on days 3, 2 and 1 with that on day 7.

B. *Transfer of plasma from normal donors to normal recipients* (Curve B, Fig. 1). Three days following transfer, the group average platelet levels were 88% ± S.D. 25% of average control value of 543,000 ± S.D. 121,000 platelets/cu mm blood. This was a significant decrease compared to the variations found in control Group A (t = 2.205;

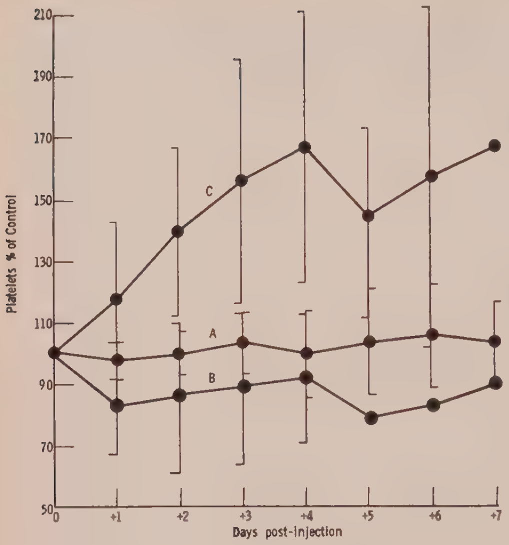

FIG. 1. Effect of plasma injections on avg platelet count of rabbits. (A) 9 uninj. rabbits; (B) 15 rabbits given plasma from normal rabbits on day 0; (C) 15 rabbits given plasma from thrombocytopenic rabbits on day 0. Brackets indicate stand. dev.

$0.05 > p > 0.02$. A significant decrease was not observed on the fourth day following transfer ($t = 1.29$; $0.3 > p > 0.2$). No difference was noted in the response of splenectomized and non-splenectomized recipients.

C. *Transfer of plasma from thrombocytopenic donors to normal recipients* (Table I; Curve C, Fig. 1). A marked increase in platelet count was observed. Comparison with the uninjected group (A) and the group receiving normal plasma (B) on the third and fourth days following transfer revealed highly significant differences ($p << 0.001$).

Discussion. Injection of plasma from rabbits made thrombocytopenic by administration of Myleran or antiplatelet serum into homologous species recipients, whether splenectomized or not, causes a marked rise in the platelet count. The duration of the response and 4-day interval between transfer and peak response suggest that the plasma stimulates production of new platelets rather than mobilization of sequestered platelets. Further experiments will be necessary to substantiate this belief. It is of interest that active plasma was obtained as early as 2 hours following production of acute thrombocytopenia by anti-platelet serum as well as after slow development of thrombocytopenia following Myleran injection. Normal plasma does not induce thrombocytosis; on the contrary, there is evidence of a significant decrease in platelet levels 3 days following injection, but not on the fourth day.[‡]

Other recent investigations have demonstrated *in vivo* transfer of a thrombopoietic factor. Keleman *et al.*(11) reported that injection of serum from thrombocythemic and some thrombocytopenic patients induced thrombocytosis of several days' duration in mice. Results, however, were variable because of the uncontrolled nature of the source. Injection of normal serum lowered the platelet count, and the effect of active test serum was counteracted by mixing it with normal human serum prior to injection.

Schulman *et al.*(12) observed that an unusual splenectomized patient with congenital persistent thrombocytopenia consistently showed thrombocytosis for more than 2 weeks following injection of normal plasma. They concluded that a factor in normal human plasma is required for platelet production, and that thrombocytopenia results from its deficiency.

Normal plasma or serum appears both to stimulate and inhibit thrombopoiesis, and regulation of platelet count may be governed by a balance between these actions. The stimulating activity can be demonstrated either when the recipient is deficient in thrombopoietic factor, as in Schulman's patient, or when a normal recipient receives plasma from thrombocythemic or thrombocytopenic donors. It remains to be determined whether the thrombopoietic effect of plasma from such donors is the result of an increased concentration of a stimulating factor or decreased concentration of an inhibitory material.

[‡] In preparation for further studies on thrombopoietin, Dr. Marjorie Zucker injected 17 additional rabbits with 25 ml plasma from rabbits made thrombocytopenic with antiplatelet serum. The average platelet count 4 days later was 121% ± S.D. 40% of the control value. This was significantly different ($p < 0.02$) from the platelet count 4 days after injection of normal plasma. The reason that the results were less striking than Dr. Spector's is not known. Odell and McDonald (Fed. Proc., 1961, v20, 51) have also reported transfer of thrombopoietin. Injection of serum from bled rats into normal rats elevated platelet counts to 160% of control value in 5 days.

Summary. Injection of plasma from rabbit donors made thrombocytopenic by Myleran or anti-platelet serum into 15 unstressed rabbit recipients raised platelet counts to 166% of control levels 4 days following transfer. These results are highly significant ($p<0.001$) when compared to the mild thrombocytopenia produced in 15 rabbits receiving plasma from normal donors or when compared to the daily variations in platelet levels of 9 uninjected rabbits. The response of normal and splenectomized recipients was similar. These results suggest that plasma contains stimulatory and inhibitory factors which regulate platelet levels.

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Comparative Reactivities of Non-fibrous and Fibrous Forms of Desoxyribonucleoprotein with Lupus Erythematosus Serum.* (26875)

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Numerous investigators have reported that factors in the sera of patients with disseminated lupus erythematosus (LE) may react with whole cells(1), isolated nuclei(2,3), desoxyribonucleic acid (DNA)(3,4,5), desoxyribonucleoprotein (DNP)(4,6,7,8), and histone(9). It has been demonstrated that there are separate components of LE sera that react with each of the "antigens". This reactivity has been demonstrated by a variety of immunologic technics including complement fixation, latex agglutination, hemagglutination, precipitation, and fluorescence microscopy. The so-called "LE factor" is reactive only with DNP and not with isolated histone or DNA(8). In detecting the anti-DNP activity of LE sera, fibrous preparations of DNP have been utilized. These forms of DNP dissociate into DNA and histone in the molar NaCl utilized in preparation, and it is not certain that the re-association product has the identical spatial association of histone and DNA present in DNP *in vivo*. Among the numerous preparations of DNP described in the literature, only one does not dissociate in molar salt solution(10, 11). No immunologic investigations are on record, so far as we are aware, utilizing non-fibrous DNP in the role of antigenic component. The purpose of the present investigation was to determine the reactivities of non-fibrous and fibrous DNP with LE sera

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by means of the complement fixation test. It was found that the non-fibrous DNP showed a much greater reactivity under the conditions of our experiments.

Materials and methods. Serum specimens were obtained from 14 patients with proven systemic lupus erythematosus. Each patient had shown a positive LE test on the day that the serum specimen was obtained. The specimens were stored at –56°C until used.

Fibrous DNP was prepared by the method of Rotherham *et al.*(12), and non-fibrous DNP by the technic of Luck *et al.*(10). In each case rat liver was employed as the original tissue. Several comments will be made about the preparation of non-fibrous DNP because it is easily converted to the fibrous form by minor variations in the preparative procedure. Non-fibrous DNP is prepared as follows. In a cold room (0-4°C) a suspension of rat liver is made in 0.002 M citric acid. This is homogenized in a low-speed Waring blendor at pH 6.1. After cellular rupture is achieved (about 3 minutes with the Waring blendor running on 45 volts) the suspension is filtered through cheese cloth and centrifuged. The supernatant and mitochondrial layers are decanted, and the nuclear sediment is washed 3 times with 0.02 M sodium acetate buffer at pH 6.1. The washed nuclei are then extracted for one hour with 0.14 M NaCl containing 0.02 M sodium citrate-citric acid at pH 6.5. After ultracentrifugation at 20,000 × g for 15 minutes the residual sediment is suspended in 1 M NaCl containing 0.02 M sodium citrate at pH 8.3. After one hour the suspension is centrifuged at 26,000 × g for 15 minutes. The supernatant is recovered and diluted with 6 volumes of distilled water, thereby precipitating the non-fibrous DNP from solution as a fine white particulate sediment. The non-fibrous DNP is redissolved in 1 M NaCl, ultracentrifuged, and precipitated as above. The DNP is suspended in 25 ml water and dialyzed overnight at 1°C against 4 liters of distilled water. The dialyzed solution is centrifuged at 26,000 × g and the supernatant is frozen in aliquots for later use. The critical points in the preparation are initial homogenization and washing of the nuclei. Homogenization must be only sufficiently long and vigorous to insure maximal cellular rupture with minimal nuclear trauma. The homogenate should be maintained at pH 6.1 ± 0.1. If the subsequent washing with acetate buffer is inadequate cytoplasmic residue will remain as a contaminant. Fibrous DNP was obtained in a number of preparations, possibly due to inadequate control at these points. In a few preparations non-fibrous DNP was noted to change to the fibrous form after precipitation of the DNP from 1 M NaCl.

The purified non-fibrous and fibrous forms of DNP, prepared as above, were made up in 1 M NaCl solutions to a concentration containing 36 μg DNA per ml, as determined by the diphenylamine reaction. Aliquots of each solution were then diluted with 1 M NaCl so that 0.1 ml would contain the equivalent of 4.5, 0.45, 0.045, and 0.0045 μg of DNA respectively (Table I). These amounts were used as "antigen" in the complement fixation test with LE serum.

The complement fixation test was performed as follows. One-tenth ml of each DNP dilution and 0.6 ml of distilled water were set up in a series of 10 × 75 mm test tubes. Two-tenths ml of LE serum (previously inactivated at 56°C for 30 minutes) was added to each tube. The tubes were left at room temperature for 20 minutes. One ml of complement (2 full units) was added and all tubes placed in the refrigerator for 16 hours, followed by 10 minutes incubation in a 37°C water bath. One-half ml of hemolysin (2 full units) was added to each tube, followed by 0.5 ml of a 2% suspension of sheep cells, and all tubes were thoroughly shaken. Simultaneous complement and serum blanks were run. After shaking, all tubes were placed in a 37°C water bath and were examined at 5-minute intervals for complete hemolysis of the complement blank. When this had occurred, the tubes were incubated 10 minutes more, then the degree of complement fixation was read as 0 to 4+, depending on whether approximately 100%, 75%, 50%, 25%, or no hemolysis was observed by comparison with appropriate controls.

TABLE I. Complement Fixation Reaction with LE Sera and Serial 10-Fold Dilutions of Non-Fibrous and Fibrous Desoxyribonucleoprotein.

	DNP concentration/tube (μg)							
	Fibrous DNP				Non-fibrous DNP			
Patient	4.5	.45	.045	.0045	4.5	.45	.045	.0045
Cd	2+	1+	0	0	2+	1+	0	0
Co	4+	1+	0	0	4+	3+	1+	0
Di	1+	0	0	0	4+	3+	0	0
Pm	4+	1+	0	0	4+	4+	3+	0
Lz	3+	0	0	0	4+	3+	1+	0
Rs	4+	1+	0	0	4+	3+	1+	0
Sv	3+	0	0	0	4+	3+	0	0

Results. Seven of the 14 LE sera demonstrated complement fixation with both forms of DNP under the conditions of these experiments (Table I). In general the reactive sera were from patients with strongly positive LE tests, and the negative specimens from patients with more weakly positive tests. A higher incidence of reactivity might have been obtained with stronger initial "antigen" concentrations of DNP, but this was not investigated. The non-fibrous DNP proved to fix complement better, in the presence of LE sera, than the fibrous DNP. This increase in reactivity lay between 10 and 100 times. There were on the other hand no obvious qualitative distinctions between the two forms of DNP in that the LE sera appeared to react with both forms of DNP or not at all.

Discussion. The present study demonstrates that non-fibrous DNP reacts with LE serum at a significantly lower concentration than the fibrous form, and thus appears to be a stronger "antigen" under these conditions. The significance of this is not clear. In general, "native" substances show stronger immunologic reactivity than their degraded or denatured counterparts; the fibrous DNP is clearly a dissociated form and possibly has been modified from its "native" state. The complex relationship between DNA and histone (13,14) may differ between the 2 forms of DNP and may have modified the spatial relationships of active sites responsible for the reaction with LE serum. However, any differences in molecular structure are apparently not great enough to abolish reactivity with LE serum. Does the anti-DNP component of LE serum arise in response to partially degenerated or altered cellular material occurring in the course of the disease If so, it might represent a secondary phenomenon. On the other hand it and similar factors may be concerned in the initiation of cellular damage. A strong reaction with a more "native" form of DNP would be consistent with the latter possibility. The question is, which form of DNP is the more "native"? This problem is further complicated by the absence of good criteria for identifying immunologically "native" DNP, and the fact that "altered" substances are sometimes more active than "native" substances(15).

Summary. The comparative reactivities of the non-fibrous and fibrous forms of desoxyribonucleoprotein were studied in the complement fixation test with LE sera. On the basis of "antigen" dilution experiments the non-fibrous DNP was shown to be between 10 and 100 times more reactive than fibrous DNP.

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Bacterial Flora of the Mouse Uterus.* (26876)

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While it is usually assumed that the uterus, like most internal organs, is sterile in the absence of manifest disease, the studies here reported indicate that microorganisms are present in the uterine cavity of a certain percentage of mice which, on surgical examination and routine histologic study, reveal no uterine disorder. A similar prevalence of endouterine organisms exists in immature mice with closed vaginas, indicating that the infection is not an ascending one.

Methods. One thousand and eighty mice of the Swiss, C57BR/cd, C57BL/6 and BALB/c strains underwent laparotomy under nembutal anesthesia. The uterus was stretched out on the tips of a forceps and the cervix was ligated. Each horn was pierced by a 25-gauge needle attached to a tuberculin syringe containing sterile broth or Ringer's Solution. One to 2 drops were injected, reaspirated and inoculated into sterile broth. In some cases, a dilution of 1:1000 of the aspirate was placed on blood agar plates. The cultures were incubated for from 24 to 48 hours and the bacteria examined on gram stained smears. In addition, a separate group of 222 C57BR/cd uteri were cultured in broth and the bacteria present in 25 of the 48 infected cultures were identified by routine bacteriological methods.† Two to 3 months later, a second laparotomy was performed and, in the BALB/c, Swiss and C57BR/cd mice, the uterine fluid which had collected was aspirated, examined by Gram stains and cultured in broth. After each laparotomy, penicillin was sprinkled into the peritoneum and achromycin was given for 3 days in the drinking water‡. The prevalence of bacteria found on broth cultures of the uteri at the first and second examinations is shown in Table I.

On the blood agar plates, the most prevalent organisms were identified§ as *E. coli, B. aerogenes, Proteus vulgaris* and *Streptococcus fecalis*, and numbers of the cultures per plate varied from 8 to innumerable. In the 25 broth cultures from C57BR/cd mice, the following bacteria were identified: *Corynebacterium hoffmannii* in 68%, *Proteus vulgaris* in 16%, *Streptococcus fecalis* in 4%, *Staphylococcus albus* in 4%, *E. coli* in 4%, and mixed infections of *Proteus vulgaris* and *Corynebacterium hoffmannii* in 4%.

All of the uteri tested showed no anomaly on clinical examination. Histologic sections were prepared on 50 random uteri and in some of them Gram stains revealed the presence of bacteria in the mucoid material adhering to the endometrium.||

The uterine fluid (Uterone) which accumulated after cervical ligation(1) in from 2 to

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† The cooperation of Sumner M. Joress, of New England Center Hospital, is gratefully acknowledged.

‡ Achromycin was graciously provided by the Lederle Co., Division of American Cyanamid, Pearl River, N. Y.

§ The cooperation of Dr. E. Kass, of Boston City Hospital, is gratefully acknowledged.

|| The histologic sections were prepared by John R. Baker, and interpreted by Dr. A. B. Russfield.

TABLE I. Prevalence of Microorganisms in Mouse Uteri and in Uterone of Hydrouteri.

Strain	Bio Swiss adults	Bio Swiss immature	BALB/c	C57BR/cd	C57BL/6
No. animals	193	75	546	181	85
No. infected (%)	14	17.3	12.6	27	35.2
Gram + cocci (%)*	74.2	38.5	55.2	44.6	23.3
Gram − cocci (%)	12.9	23	15.6	21.6	0
Gram + rods (%)	3.2	7.7	10.4	8	13.4
Gram − rods (%)	9.7	23.0	15.6	21.6	63.3
Others (%)	0	7.7	3.2	4.1	0
% Uterone containing bacteria	32	—	17.5	49	—
Gram + cocci (%)†	23	—	22	39	—
Gram − cocci (%)	23	—	18	22	—
Gram + rods (%)	19	—	9	4	—
Gram − rods (%)	27	—	51	35	—
Others (%)	7.6	—	0	0	—

* On smear made from broth culture (% refers to No. of uteri).
† On smear made from uterine secretions (% refers to No. of uteri).

3 months contained bacteria in a higher percentage of cases than were found at the first laparotomy. The nature of the bacterial flora in Uterone was different from that found at first exploration, in that the prevalence of Gram-positive cocci was markedly reduced and that of Gram-negative cocci was significantly increased. Whether this change in bacterial population is due to the brief postoperative antibiotic therapy or to other factors such as, possibly, lysozyme which has been demonstrated in Uterone(2) remains to be determined. The majority of the uteri which contained bacteria at time of uterine ligation remained contaminated, showing bacteria in the accumulated Uterone (79% of the 202 BALB/c; 69% of the 54 Bio Swiss; and 50% of the 20 C57BR/cd mice). Only 24 animals out of a total of 274 showed a change from an initially contaminated uterus to sterile luminal fluid; on the other hand, 44 mice shifted from initially sterile uteri to contaminated Uterone. This higher rate of bacterial contamination in hydrouteri, while possibly due to contamination at the time of puncture, may be analogous to the well-known high frequency of clinically manifest uterine infection in mice receiving estrogen.

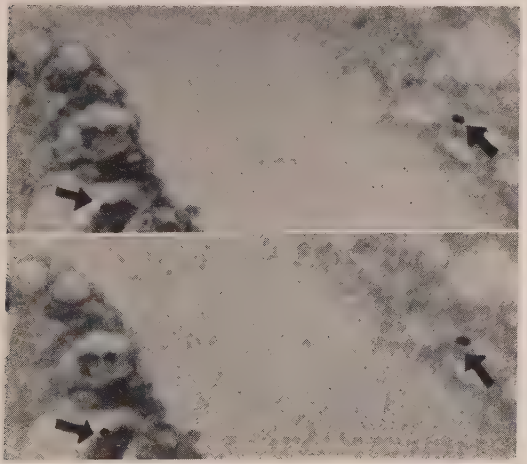

FIG. 1. Mouse uterus. Arrows point to Gram-positive cocci in intrauterine secretions (right) and in endometrial epithelial cell (left). Top and bottom photographs show the same field but in slightly different focus. Arrows correspond to about 5 μ in length (Gram stain, × 1000).

Discussion. The demonstration of bacteria in the uteri of immature and mature mice based on direct histologic examination of uteri and on culture of uterine washings may have far-reaching biologic significance. It renders necessary a critical reevaluation of studies on the chemistry and biologic activity of endometrial secretions(3) which may, in part, be properties of bacterial products or influenced by them. It raises the question whether the embryo, during even the earliest phases of pregnancy may, contrary to general belief, develop within an environment which is complicated by the presence of bacteria. This may have an important bearing on the physiology of cesarean section-derived experimental animals. It may also be a factor in causation of fetal anomalies. Responses of the uterus to estrogenic and progestational hormones may be the end result of interaction between hormones, target and bacteria. Finally, it would appear that Uterone may contain substances which favor growth of some bacteria while inhibiting others.

Summary. Microorganisms were demonstrated by direct smears and cultures of uterine fluid in immature and mature mice and in uterine tissue sections. Incidence of uterine contamination varied from strain to strain. Contamination persisted in the luminal fluid of hydrouteri. However, Gram-negative bacteria increased and Gram-positive organisms decreased during accumulation of uterine fluid.

The technical assistance of Suzanne Warner is gratefully acknowledged.

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Stimulation of Progestin Release from Rabbit Ovary *in vivo*.* (26877)

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The principal functional roles ascribed to progesterone have been related to pregnancy, and the primary site of synthesis of this steroid has been considered to be the corpus luteum of the ovary. This would imply that ovarian progesterone is not released in the absence of functioning corpora lutea. However, Forbes(1) detected elevations in progestational activity of peripheral blood drawn from female rabbits shortly after mating, several hours before ovulation could be expected; and Edgar(2) reported that the progesterone content of ewe ovarian vein blood could not be correlated with the number of functioning corpora lutea in the cannulated ovary. Zander(3) found high concentrations of progesterone in the human Graafian follicle shortly before ovulation and also isolated the progesterone metabolite, Δ^4-3-ketopregnene-20 α-ol (20α hydroxy-pregn-4-en-3-one) from the mature human follicles(4). Recently Taylor(5) has demonstrated the presence of appreciable progesterone in ovarian tissue of immature rats. Progesterone and its α 20-ol metabolite have also been found in the adrenal venous blood of calves of both sexes(6).

This paper gives further evidence that release of progesterone and Δ^4-3-ketopregnene-20 α-ol from rabbit ovaries *in vivo* is independent of the presence of corpora lutea, and that release of these steroids is rapidly activated following coitus or injection of hormonal agents which induce ovulation. Rabbits were chosen for this study since this species ordinarily ovulates only in response to coitus, and presence or absence of corpora lutea can easily be controlled.

Materials and methods. Steroid assays were made of ovarian blood of more than 60 New Zealand white rabbits. Sexually mature females (3.5-5 kg) were anesthetized with pentobarbital (approx. 25 mg/kg body wt), and 500 USP units/kg heparin were injected *i.v.* immediately before a polyethylene cannula (o.d. = 0.060″) was inserted into the ovarian vein. Blood samples were collected for specific time intervals in order that progestin content could be expressed in units

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of output; *i.e.*, μg/gland/hour.

Following initial collection of a control sample, hormones were administered intravenously in the following dosages: luteinizing hormone (LH) 1-5 Armour units; follicle stimulating hormone (FSH) 2.25 and 5.0 Armour units; lactogenic hormone (LGH) 200 I.U.; growth hormone (STH) 5 U.S.P. units;‡ thyroid stimulating hormone (TSH) 10 U.S.P. units;§ corticotropin (ACTH) 4 I.U.;‖ chorionic gonadotropin (HCG) 250 I.U.;¶ and pregnant mare's serum (PMS) 250 I.U.** To prevent shock from blood loss, alternate collections of 10-15 minutes duration were reinjected and blood volume was maintained by addition of physiological saline or 5% dextrose in saline.

Each blood sample was measured, hemolyzed with distilled water and extracted overnight with 3 volumes of ether and twice the next morning with 2 additional 50 ml of ether. The final ether extraction and blood buffer coat were washed twice with 0.1 N NaOH and distilled water. The 3 ether washings were then combined and evaporated to dryness, and the remaining residue was partitioned twice between 10 ml absolute methanol and 10 ml n-heptane. The methanol phase, which now contains the steroid, was evaporated to dryness and the residue redissolved in 1 ml ethyl acetate, half of which was chromatogrammed on Whatman #1 paper strips 7 × 22 inches. A descending system was used. Strips were equilibrated overnight at room temperature in a closed tank containing approximately 500 ml of stationary phase, Methanol:water::4:1. The mobile phase, n-heptane:ethyl acetate 5:2 was added the next morning.

As standards, progesterone 5, 10, 15 μg and Δ^4-3-ketopregnene-20-ol†† 50, 75 and 100 μg were also applied to the paper and run with each blood assay. After development (3-4 hours) purple fluorescent spots were identified with a Mineralight UV lamp and the paper was sprayed with a filtered solution of 2,4-dinitrophenylhydrazine in 1% HCl in ethanol. After washing in warm tap water to remove background color, strips were dried and steroid areas were located against the standards, cut out and eluted in 2 successive half-hour ethanol washings (with frequent shaking) of 6 and 4 ml respectively. Color was developed by addition of 0.5 ml 2 N NaOH to the combined washings and read in the Klett colorimeter (540 green filter) with the paper background blank set at zero. Criteria of progesterone identification were 1) solvent partitioning, 2) UV fluorescence and 3) paper chromatography.

Results. On our solvent system, progesterone and its 2 active metabolites, Δ^4-3-ketopregnene-20 β-ol and Δ^4-3 ketopregnene-20 α-ol, had chromatogram Rf values of 0.6, 0.48 and 0.35 respectively. Whereas Δ^4-3-ketopregnene-20 β-ol was not found in rabbit ovarian vein blood, appreciable output of Δ^4-3-ketopregnene-20 α-ol, as identified by Rf value ranged from 50-100 μg/gland/hour, and small amounts of progesterone could frequently be detected in 15 or 20 minute blood collections from non-pregnant, estrous rabbits without corpora lutea.

Within minutes following mating (4 cases) or injection of a gonadotropin which induced ovulation (LH, 5 cases; FSH, 5 cases; PMS, 5 cases; HCG, 8 cases) progestin levels rose abruptly, often to exceed an output of 1 mg/gland/hour (Fig. 1). Similar results were encountered following injections of TSH (Armour 216-174-1 and Thytropar) which were obviously contaminated with gonadotropin since ovulation ensued. A highly purified TSH preparation§ which induced thyroid stimulation without concomitant ovulation was also ineffective in stimulating progestin release. This output of progestin was independent of follicular rupture since ovulation in the rabbit follows 15-20 hours after the ovulating stimulus. Administration of pituitary hormones which did not induce ovulation (*i.e.*, ACTH, 8 cases; TSH, 2 cases; STH, 2 cases; LGH, 10 cases) caused no

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‡ All by courtesy of J. D. Fisher, Armour Pharmaceutical Co.

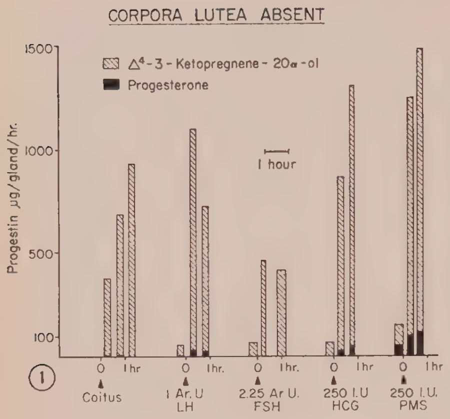
§ Courtesy of Drs. John Pierce and Mary Carsten, Department of Physiological Chemistry, UCLA Medical School.

‖ Parke-Davis.

¶ "Follutein," Squibb.

** "Equinex," Ayerst.

†† Courtesy of Dr. D. Perlman, Squibb Inst.



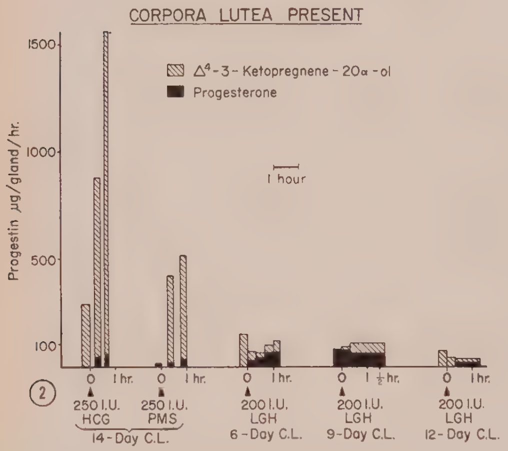


FIG. 1. Typical effects of coitus and treatment with gonadotropins on progestin release from nonluteinized rabbit ovary. The width of each bar indicates duration of ovarian blood collection for that sample.

FIG. 2. Typical effects of treatment with placental gonadotropins and lactogenic hormone (LGH) on progestin release from rabbit ovary during pregnancy or pseudopregnancy.

significant changes in progestin release. Similarly no progestin release was recorded in 2 cases of coitus followed by failure of ovulation and one case of a non-ovulatory injection of LH in a sexually immature rabbit.

In the pregnant or pseudopregnant rabbit with vascularized corpora lutea (14 day) HCG and PMS both effectively stimulated progestin release. This was in contrast to LGH which, in doses of 200 I.U., was consistently ineffective (Fig. 2).

Discussion. The principal progestin found in the ovarian vein blood of adult New Zealand white rabbits is Δ^4-3-ketopregnene-20 α-ol. This biologically active metabolite of progesterone (1/3-1/2 as active as progesterone in the rabbit uterus test) has been found in high concentrations in human Graafian follicles and placenta and is oxidized to progesterone *in vitro* by corpora lutea or in ovarian tissue without corpora lutea(4).

The rapid elevations in progesterone and Δ^4-3-ketopregnene-20 α-ol release from the ovaries of estrous rabbits following gonadotropic stimulation show clearly that functional corpora lutea are not essential to the elaboration of these steroids. The results also suggest that lactogenic hormone (LGH) may not be luteotropic in the rabbit, confirming the proposals of Klein and Mayer(7) and others in this respect.

A probable precursor of ovarian hormones has been suggested by Claesson, Hillarp and Högberg(8), who noted a marked fall in the esterified cholesterol of rabbit interstitial tissue shortly after administration of gonadotropin. Although these workers concluded that cholesterol must be the principal precursor of ovarian estrogen, our results imply that the rapid changes in interstitial gland cholesterol may be correlated with progestin output.

Summary. 1) Progesterone and its metabolite Δ^4-3-ketopregnene-20 α-ol are present in the ovarian vein blood of estrous rabbits without functional corpora lutea. 2) After mating or administration of a gonadotropin which induces ovulation, the output of these steroids is immediately elevated. 3) Gonadotropins, but not lactogenic hormone (LGH), also activate progestin release from the ovary containing functional corpora lutea of pregnancy or pseudopregnancy.

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Alcohol and Caffeine in Choice-Discrimination Tests in Rats. (26878)

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It is a popular belief that caffeine is an effective antagonist to alcohol. This is undoubtedly based on the generalization that alcohol is a central depressent while caffeine exerts a stimulant action centrally. Despite the apparent "stimulation" from alcohol which is evident in some persons, biological scientists agree that, if classification is essential, ethanol must be categorized as a depressant.

However, both hypnotics and tranquilizers are also central depressants. The mechanism of action, herein, as well as end results are very different and terminology such as stimulant or depressant drug is becoming meaningless.

Popular usage of the term tranquilizer often suggests that alcohol has such properties. This has been challenged since a tranquilizer should reduce anxiety without greatly obtunding other behavioral responsiveness (1). We have shown that alcohol will diminish anxiety but does so only at the expense of various parameters of performance and behavior(2,3,5).

This study was begun in order to measure the effect of alcohol-caffeine combinations on performance in the rat. It was anticipated that caffeine might reverse some of the effects of alcohol since this is so commonly reported.

Materials and methods. The experimental technique employed a shock-avoidance box (Fig. 1) in which a choice must be made for escape(1,2,3,4). The floor of the main compartment (A) consists of stainless steel rods. A warning light (L) serves as a conditioning stimulus and is presented 5 seconds before a shock is delivered *via* the grid flooring. The door (D) is lifted simultaneously with the warning light to expose the escape compartments (B) and (C). If the rat fails to escape, 5 seconds later he receives a 5-second shock delivered by the grid floor. The rat may escape to a *safe* and darkened area (B) with insulated non-shock floor, or to an *unsafe* illuminated area (C) with a grid floor that will deliver shock.

The only animals used in this study were those that had learned to respond with 100% accuracy to the "anxiety-producing" warning light by escaping to the *safe* area (B), thus avoiding shock. When the rat observes and reacts to the warning light, we consider this to be a simple conditioned avoidance response (CR). If the rat fails to leave the main compartment during the 5 seconds in which the light is on, a shock of 15v A.C. from a relatively low resistance power supply is applied to the grid for 5 seconds. If this is effective and the animal leaves the main compartment, this is recorded as an unconditioned avoidance response (UR). With our apparatus, the rat must make a decision-choice (discrimination) of the correct compartment into which he must flee. We recorded errors in discrimination as incorrect choices of compartment after a stimulus of light or of shock.

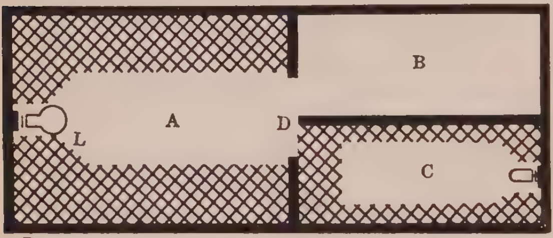
Weanling male rats were trained 3 times daily and at each testing period received 3 trials. With this 9-trial daily testing period, 90 to 95% of the animals learned to escape properly with 100% accuracy in 4 days.

All drugs were administered intraperitoneally. Alcohol was administered as a 5% solution. Caffeine was administered as the citrate salt but calculated as 50, 100 or 150 mg/kg of body weight of the base.

After administration of the alcohol, caffeine or alcohol-caffeine combinations, the animals were tested 3 times every 15 minutes for 4 hours thereafter. Dosage levels were: 50 mg/kg, 100 mg/kg, and 150 mg/kg of caffeine, 1 g/kg of ethanol, and 1 g/kg of ethanol combined with 50, 100, or 150 mg/kg of caffeine. A control group of rats received only saline injections. Each of the total of 90 rats was used for only one 4 hour test and discarded at the end of the test period. The dosage of ethanol was selected because previous reports from our laboratory indicated that this induces only a moderate effect on diminishment of performance(3). Larger doses may cause gross immobility of the animal. Preliminary studies indicated that 5, 10 or 25 mg/kg of caffeine effected no significant change in responsiveness in the animals. Therefore, 50 mg/kg of caffeine was used as the low dosage level.

Results. Total scores for the animals for each hour are represented graphically in Fig. 2, 3, 4. The control group of 10 rats continued to respond correctly, *i.e.*, no suppression of total conditioned responses. A small dose of caffeine caused no significant reduction in conditioned responses. The intermediate dose (100 mg/kg) effected a moderate reduction in conditioned responses for the first 2 hours only. A large dose (150 mg/kg) of caffeine caused a marked reduction in CR which lasted longer than the 4 hour period. Alcohol alone (1 g/kg) caused a slight (5%) reduction of CR during the first hour. However, when alcohol was combined with caffeine, the rats were markedly affected in that a greater reduction in conditioned responses resulted than could be explained by addition of the 2 drugs. There was no drug antagonism demonstrated by this "stimulant-depressant" combination. Thus, potentiation was most evident when an intermediate dosage of caffeine was combined with alcohol.

A like set of results was obtained for the



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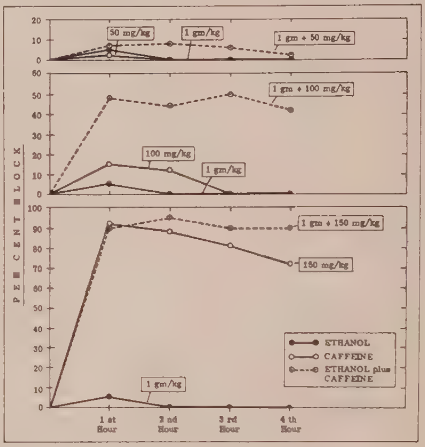

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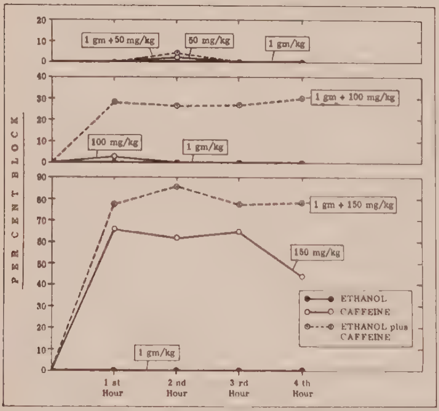

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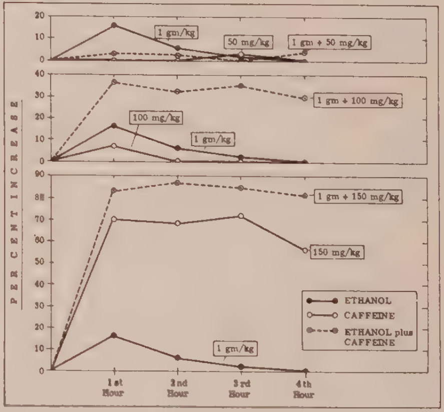

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FIG. 1. Apparatus and training method. A, main compartment: warning light, shocking grid floor. B, safe escape area: not illuminated, insulated floor. C, unsafe escape area: illuminated, shocking grid floor. D, Guillotine-type door. L, warning light.

FIG. 2. Suppression of conditioned responses in rats after alcohol, caffeine and alcohol-caffeine combinations.

FIG. 3. Suppression of unconditioned responses in rats after caffeine, alcohol and caffeine-alcohol combinations.

FIG. 4. Increase in errors in choice-discrimination in rats after alcohol, caffeine and alcohol-caffeine combinations.

unconditioned response (Fig. 2). Once again alcohol-caffeine combinations gave greater depression of responses than simple addition can explain.

Similarly, an increase in errors of choice of exits (discrimination) was observed after caffeine and alcohol were combined (Fig. 4). The rats were not noticeably stimulated by 50 or 100 mg/kg of caffeine or depressed by the alcohol alone. However, when 100 mg/kg of caffeine and 1 g/kg of ethanol were administered to the animals, an obvious depressed state resulted. The animals receiving this combination were able to respond to the unconditioned shocking stimulus but did so in a confused manner and with a large number of errors. This potentiation was also evident in the animals' receiving larger doses (150 mg/kg) of caffeine.

Discussion. An intraperitoneal injection of 1 g/kg of ethanol will effect a peak blood level in rats of approximately 90 mg% in 15 minutes and this will have largely disappeared from the blood within 2 hours(3). In the present study the effect of alcohol had disappeared by the second hour. This dose of alcohol did not produce behavioral changes such as drowsiness, reduction in activity or aggressiveness. Caffeine in a dosage of 100 mg/kg i.p. altered behavior which returned to normal at the end of 2 hours. With this dosage of caffeine, some of the animals showed some hyperexcitability when handled.

The above dose of alcohol (1 g/kg) combined with caffeine (100 mg/kg) produced an effect in behavioral responses which was still present at the end of 4 hours. These animals were grossly depressed. Activity was not measured but was obviously reduced at the end of 4 hours, although the animals that received either drug alone appeared to be unaffected. The degree of gross depression seems to correlate with the reduction in behavioral responsiveness measured. It is apparent from the data that alcohol altered the behavior in such a manner that recovery was not complete although blood levels might indicate otherwise. There is evidence that recovery had scarcely begun when zero blood levels of alcohol had been reached.

The same potentiation is apparent when higher dosages (150 mg/kg) of caffeine were used but this concentration of the alkaloid is grossly depressant by itself.

It was not evident from our studies that caffeine antagonized alcohol. Rather it appears that caffeine potentiates the slight depression of conditioned and unconditioned responses due to this dosage of alcohol as well as markedly increasing errors in discrimination.

Studies in human subjects are in progress to test the hypothesis that alcohol produces behavioral degeneration which remains long after the alcohol has disappeared from the blood and that certain drugs, including stimulants, may exaggerate this effect.

Summary. A series of rats received alcohol, caffeine, or alcohol-caffeine combinations. Conditioned and unconditioned responses as well as erors in choice (discrimination) were measured. No significant changes were noted in total conditioned or unconditioned responsiveness with small doses of caffeine (50 mg/kg) or with a dosage of 1 g/kg of ethanol. By all measurements, as well as observation, caffeine potentiated the depressive effect of alcohol long after the alcohol had disappeared from the blood. This was most marked with intermediate (100 mg/kg) or with high (150 mg/kg) dosages of caffeine. In no instance did caffeine antagonize the effects of alcohol.

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Effects of Radiation on Tissue Serotonin Levels in the Rat.* (26879)

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It has been demonstrated by a number of workers that serotonin (5-hydroxytryptamine) has marked radioprotective activity when injected prior to x-irradiation and on a weight basis is one of the most potent radioprotective agents known(1-5). Since serotonin is a normal constituent of body tissues it was of interest in view of the above to inquire whether the serotonin content of an animal's tissues was related to its capacity to withstand radiation injury and further what effect exposure to radiation would have on tissue serotonin levels. Data are presented here on the effects of total body x-irradiation and gamma radiation on tissue serotonin levels in the rat.

Procedure. Thirty-two male rats of the Holtzman strain which had been raised from weaning on a natural food stock ration with an average body weight of 451 (range 411 to 530) g were used. Animals were placed in individual metal cages with raised screen bottoms and were divided into 4 comparable groups of 8 each. Group I was exposed to a single dose of 900 r total body x-irradiation; Group II was administered continuous total body gamma radiation at a level of 500 r per week; Groups III and IV served as untreated controls. Rats in Groups I, II and III were fed *ad libitum*‡ with food consumption determined daily for animals in Groups I and III. Those in Group IV were pair-fed daily an amount of food equal to the average of that ingested the previous day by animals in Group I. Water was provided *ad libitum* to all groups. Six days after x-irradiation rats in Groups I, III and IV were mechanically stunned and after withdrawal of 1 ml of cardiac blood with a heparinized needle, killed by decapitation and serotonin content of blood and tissues determined according to the method of Bogdanski *et al.*(6). Radiation factors employed for Group I were as follows: GE model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter§; HVL, 2.15 mm Cu; target distance to top of box, 78 cm; and exposure dose rate, 17.92 r/min (measured in air, at top of box). The animals to be irradiated were placed in a wooden box divided into 14 compartments 7 cm wide, 16 cm long and 10 cm deep. The partitions and top were made of ⅛-inch cellulose acetate sheeting; and the top, one side and bottom of each compartment were perforated with holes for purposes of ventilation. The gamma radiation administered to rats in Group II was supplied by a Cesium-137/Barium-137 source mounted in a camera.‖ Metal cages with raised screen bottoms were grouped around the camera so that each cage received an average exposure of approximately 500 r/wk. Animals were continuously exposed to radiation except for a short period (approximately 10 minutes) each day at which time they were fed and watered. Rats in Group II were sacrificed after 9 days of gamma radiation and serotonin content of blood and tissues determined as indicated above.

Results. Findings (Table I) indicate that a single dose of 900 r total body x-irradiation resulted in a highly significant reduction in serotonin concentration of the blood and spleen and a marked increment in serotonin concentration of the small intestine compared to values obtained in untreated *ad libitum*-

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† With Neurochemical Research Laboratory, Veterans Admin. Hospital, Sepulveda, Calif. Present address: Dept. of Psychiatry, Univ. of Iowa School of Med., Iowa City.

‡ Rockland Rat Diet, A. E. Staley Manufacturing Co., Decatur, Ill.

§ A nonuniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand(11). The center of the filter had a thickness of 1.7 mm Cu; the edge, 0.5 mm Cu.

‖ The gamma radiation facilities were designed and constructed by Isotopes Specialties Co., Burbank, Calif.

TABLE I. Effects of Radiation on Tissue Serotonin Levels (8 Rats per Group).

Group	Body wt, g		Serotonin content of following tissues				Spleen wt, mg	Total serotonin content of spleen, μg
	Initial	Final	Blood, μg/ml*	Spleen, μg/g*	Small intestines, μg/g*	Brain, μg/g*		
I (X-irradiated)	453.6	391.9	.21 ±.03	1.21 ±.11	5.47 ±.35	.59 ±.06	516	624
II (Gamma-irrad.)	450.4	416.4	.89 ±.10	1.57 ±.19	3.03 ±.17	.58 ±.05	767	1204
III (Untreated, fed *ad lib*)	449.7	454.5	.87 ±.07	2.63 ±.19	3.38 ±.25	.81 ±.05	1187	3122
IV (Untreated, pair-fed to Group I)	449.4	392.0	.68 ±.17	2.13 ±.16	3.55 ±.15	.58 ±.04	824	1755

* Including stand. error of mean.

fed and pair-fed controls. Since x-irradiation also caused a highly significant reduction in average weight of the spleen, total serotonin content of this organ relative to that of the untreated controls was even more depressed than was indicated by data on the serotonin concentration per gram of tissue alone. A significant reduction in both concentration and absolute amount of serotonin in the spleen was also observed in rats exposed to continuous gamma radiation but to a lesser degree than in the x-irradiation series. In contrast to findings in the latter group, serotonin concentration in the blood and small intestine of rats exposed to gamma radiation did not differ significantly from that of untreated controls. Brain serotonin content was also reduced in x-irradiated and gamma-irradiated rats compared to values in untreated *ad libitum*-fed controls.¶ A similar reduction was also observed, however, in untreated, pair-fed controls.

Discussion. A number of investigators have reported that the spleen plays an important role in resistance to x-irradiation (see review of literature by Jacobson and coworkers(7,8)). Shielding of the spleen during irradiation, the intraabdominal implantation of spleen from normal animals into irradiated animals after exposure, intraperitoneal injections of isologous spleen cells or spleen homogenate or of homologous and heterologous cell-free spleen extracts have all been reported to reduce mortality following exposure to a lethal dose of total body x-irradiation in various species of animals including the mouse, guinea pig, rabbit and dog although there is some evidence that such procedures are less protective in the rat(9). The marked reduction in serotonin content of the spleen of both x-irradiated and gamma-irradiated rats in the present experiment might possibly be related to the findings indicated above. It is well established that the serotonin in blood is present primarily in the platelets and that the latter are significantly reduced following exposure to doses of x-irradiation comparable to that employed in the present experiment(10). A reduction in blood serotonin concentration might have been anticipated, therefore, on the basis of the reduced platelet count and absence of binding sites for serotonin in the blood. The increased concentration of serotonin in the small intestine of x-irradiated rats might reflect a defect in the transport mechanism of serotonin from the small intestine related possibly to the reduction in blood platelet count. No data are available as to the significance of the changes noted above. It is possible that reduction of tissue serotonin levels in the blood and spleen following x-irradiation is directly related to the pathological manifestations of radiation injury. If so, determining serotonin content of blood or spleen may be a sensitive index of radiation injury and a useful tool for rapid evaluation of radioprotective agents. Further studies are indicated to determine the time-dose relationship of tissue serotonin levels following radiation and the effects of radioprotective agents thereon.

¶ Values for brain serotonin concentration in this study which were obtained on animals approximately 10 months of age at time of sacrifice were higher than those previously reported in younger rats of another strain(12). To determine the effects of age on brain serotonin levels, determinations were made of brain serotonin concentration of 8 male rats of the Holtzman strain obtained from the same breeding colony as the older animals and raised from weaning on the same stock diet. Animals were sacrificed at 33 days of age and an average body weight of 134 g and brain serotonin levels determined by the same procedure as was employed for the older animals. Serotonin concentration in the brain of the younger animals averaged .28 ± .02 μg/g. It would appear that brain serotonin concentration increases with age, this agrees with findings previously reported by Kato(13).

Summary. Rats exposed to a single dose of 900 r total body x-irradiation exhibited a highly significant reduction 6 days after x-irradiation in the serotonin concentration of the blood and spleen and a marked increment in serotonin concentration of the small intestine compared to values obtained in untreated *ad libitum*-fed and pair-fed controls. A significant reduction in serotonin concentration in the spleen was also observed in rats exposed to gamma radiation. No significant difference in brain serotonin concentration was observed between x-irradiated and gamma-irradiated rats and untreated pair-fed controls with values for all 3 groups, however, reduced below that of untreated, *ad libitum*-fed controls.

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Nicotine Oxidation by *Arthrobacter*.* (26880)

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The metabolism of nicotine by several micro-organisms has been investigated and a number of oxidation products of the alkaloid have been isolated and identified. These products include 6-hydroxynicotine, 6-hydroxypseudooxynicotine, pseudooxynicotine and a group of derivatives in which the pyrrolidine ring of nicotine has been opened and degraded. Rittenberg and co-workers(1,2,3) have obtained cell-free preparations of a soil bacterium, designated as P-34, which catalyzed the oxidation of nicotine successively to 6-hydroxynicotine, 6-hydroxypseudooxynicotine and 2,6-dihydroxypseudooxynicotine. Wada(4,5) reported that a Pseudomonas species, also isolated from soil, could oxidize nicotine to some derivatives in which either the pyridine or pyrrolidine ring or both were oxidized. Frankenberg and Vaitekunas(6) have reported similar nicotine metabolites to be produced by unidentified organisms derived from the surface of tobacco seeds. Wadsworth(7) has shown that a Corynebacterium species can convert nicotine to a compound resembling 6-hydroxynicotine. Sguros(8) and Hylin(9) have described the cultural and morphological characteristics of *Arthrobacter oxydans,* an organism obtained from air and tobacco leaves, which has the ability to catalyze the oxidation of nicotine. The identity of the oxidation products was not established, however. Decker and coworkers(10) have shown that oxidation of nicotine by a cell-free extract of *Arthrobacter oxydans* proceeds with the formation of 6-hydroxynicotine.

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* This work was taken in part from a thesis by Gail D. Griffith in partial fulfillment of the requirements for the Ph.D. degree at Michigan State University. The work was supported by a research grant from Nat. Inst. Health.

Although 6-hydroxynicotine has been identified as a nicotine metabolite in several types of bacteria, the responsible organisms have not been adequately described in any case. The present paper presents evidence for the formation of 6-hydroxynicotine as an oxidation product of nicotine produced by a species of *Arthrobacter* present on the roots of tobacco plants.

Methods. Isolation and characterization of the microorganism. During a study of nicotine metabolism in the tobacco plant it was noted that washings of tobacco roots had the ability to transform nicotine to a product having an ultraviolet absorption spectrum markedly different from that of the parent compound. The tobacco plants had been raised from seed in Vermiculite† in a greenhouse and were subsequently brought into the laboratory and grown in inorganic nutrient solution(11). Two microrganisms were observed in gram stained preparations and under a phase contrast microscope in the sediment of nutrient solution in which plants had been growing. The first was a non-motile gram negative organism, with darker staining granules, which existed in several morphologies from coccoid to bacilliform. The second was a gram negative, motile, short, slender bacillus. This organism was observed later in mixed colonies with the first organism, but was never isolated.

The first organism was isolated using the nicotine-salt-yeast extract liquid and agar media described by Sguros(8). The liquid medium was also used for observation of the organism's life cycle and nicotine-degrading activity. All cultures were incubated at 24-26°.

Results. In several respects the organism was similar to *Arthrobacter oxydans*(8). It exhibited a life cycle which was complete in 29 hours in cultures grown with aeration. Aged cultures were in the coccoid cystite form; within 4 hours after inoculation into fresh liquid medium the cystites formed one to 3 germ tubes emerging at obtuse angles to one another. Two or more organisms were often seen linked together in a chain-like arrangement. After 12 hours the organisms appeared to be long bacilli with a number of darker staining granules apparent within them. At about 17 hours the organisms were shorter and the granules were more diffuse, ellipsoidal in shape, and less well defined. At 24 hours the granules again became more spherical and distinct. Finally at 29 hours the organism assumed a homogeneous morphology consisting of cocci, or crystites, singly, in pairs and in clusters as observed in stained preparations.

Crystites were largely gram positive with very little gram negative material apparent within the cells. The germ tubes were faintly gram negative. As the cells matured, they became more strongly gram negative with the granules appearing as darker-staining bodies. At about 26 hours the cells contained distinctly gram positive granules and at 29 hours again had assumed an almost completely gram positive coccoid form.

The organism produced a deep blue diffusible pigment first visible at about 4 hours in aerated liquid cultures. The chemical nature of this pigment was not studied. The intensity of the color increased until at 24 to 36 hours the cultures assumed a blue-black appearance. Upon further aging of the cultures the color became yellow-brown. The production of a blue pigment has also been reported for other nicotine-oxidizing bacteria (2,8,10).

Growth on nicotine-containing agar plates produced small, raised, gray-white mucoid and slightly opalescent colonies. Older colonies exhibited a dense diffusible blue pigment which became yellow-brown in about 10 days.

Proteolytic activity of the organism was demonstrated by its ability to liquefy gelatin.

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† Commercial heat-expanded mica.

Liquefaction began at the surface of the medium and proceeded to about ½ the depth of the tube in 10 days and proceeded no further.

Nicotine oxidizing activity of the isolate under consideration was greatly diminished when the cultures were not aerated during incubation, implying a dependence on molecular oxygen.

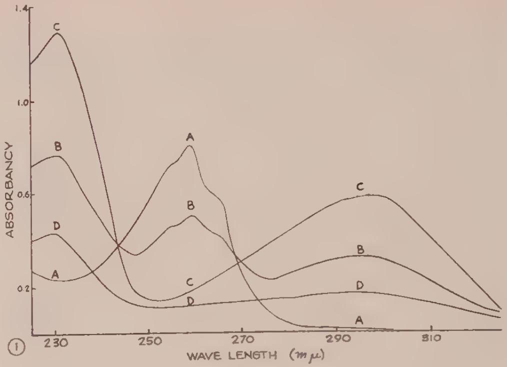
Application of 3% hydrogen peroxide solution to colonies of the organism on nicotine agar plates resulted in immediate vigorous formation of gas, indicating catalase activity.

The isolated organism differed from *Arthrobacter oxydans* in one respect: sucrose and fructose support growth of the organism but no acid or gas are produced either in static or aerated cultures. It has been reported that *Arthrobacter oxydans* produces acid from these substrates(8). Possibly then the isolate under consideration represents a unique species or strain.

Nicotine oxidation. During the various stages of the life cycle of the organism samples of the cultures were taken, and inactivated by addition of a 10% aqueous solution of trichloroacetic acid followed by heating for 5 minutes in a boiling water bath. Insoluble material was removed by centrifugation and aliquots of the samples were assayed on a Cary recording spectrophotometer in the ultraviolet range (220 to 340 mμ). The spectra of several of these samples are shown in Fig. 1.

Nicotine and 6-hydroxynicotine concentrations at different stages in the growth of the organism (Fig. 2), were calculated from molecular extinction values and absorbancies at their characteristic absorption maxima (259 mμ for nicotine, 295 mμ for 6-hydroxynicotine). The nicotine concentration remained relatively constant for 13 hours, after which it rapidly dropped to zero. Formation of 6-hydroxynicotine began at 13 hours. Its concentration reached a maximum at 16.5 hours, then the compound rapidly disappeared. Maximum concentration of 6-hydroxynicotine in the medium was approximately 50% of the starting nicotine concentration on a molar basis, as shown in Fig. 2.

Isolation and Identification of 6-Hydroxynicotine. The procedure used for isolation of 6-hydroxynicotine was essentially that described by Hochstein and Rittenberg(1). Two ml of *l*-nicotine in 1.5 l of inorganic nutrient solution(11) were inoculated with organisms, and after 4 days incubation with aeration, 6-hydroxynicotine concentration had reached a maximum. The extended period required for completion of nicotine oxidation in comparison with the studies already described was presumably the result of differences in pH and composition of the medium. The solution was made 0.1 *N* with HCl and heated on a steam bath for 15 minutes. The insoluble material was removed by filtration. A saturated aqueous silicotungstic acid solution was added to the filtrate until no more precipitate formed. After standing overnight, the silicotungstic acid salt was removed by filtration and decomposed with $Ba(OH)_2$.



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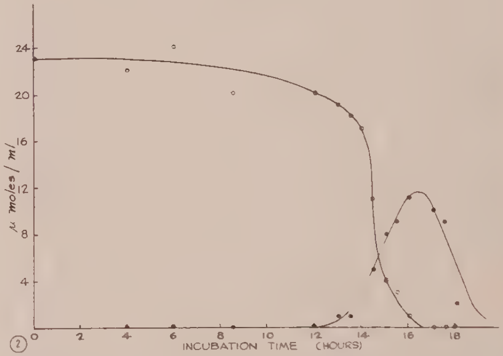

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FIG. 1. Ultraviolet absorption spectra showing oxidation of nicotine to 6-hydroxynicotine by an Arthrobacter species in aerated liquid cultures. A, Zero time (nicotine); B, 14.5 hr incubation; C, 17.0 hr incubation; and D, 18.0 hr incubation.

FIG. 2. Relative concentrations of nicotine and 6-hydroxynicotine during oxidation by Arthrobacter in aerated liquid cultures. ○——○, nicotine; ●——●, 6-hydroxynicotine.

Excess Ba^{++} was removed as $BaCO_3$. The filtrate was decolorized with a small amount of Norite and evaporated to dryness under reduced pressure at 40°. The solid residue was extracted with boiling ligroin (b.p.60-90°). The product was allowed to crystallize from the solution and was recrystallized several times from this solvent; melting point, 120.5-121.5, melting point of the picrate, 165.5-166, molecular extinction (in 0.1 N HCl) 5440 at 295 mμ and 11,700 at 232 mμ, and a specific rotation (1.5% aqueous solution) of –68.9°.

These data may be compared with similar physical constants for 6-hydroxynicotine (1,10).

A mixed melting point of the picrate prepared from the isolated product with that of the picrate prepared from 6-hydroxynicotine‡ was not depressed.

An elementary analysis of the isolated product gave the following results for $C_{10}H_{14}ON_2$§: Found—67.44% C, 7.90% H, 15.8% N, Calculated—67.42% C, 7.86% H, 15.73% N.

The infrared spectrum of the isolated compound in carbon tetrachloride solution was identical with that of 6-hydroxynicotine.

Discussion. The ultraviolet absorption spectra presented in Fig. 1 show that the characteristic peaks of 6-hydroxynicotine are retained until the ultraviolet absorption disappears. This seems to indicate that no other ultraviolet absorbing compound was produced by the organism. The second product in the metabolic pathway might then be one in which the pyridone ring of 6-hydroxynicotine is altered, since this part of the molecule is largely repsonsible for the ultraviolet absorption. However, since intact cells were used in these experiments no definite conclusion can be drawn. Perhaps the enzyme system catalyzing the conversion of nicotine to 6-hydroxynicotine was present on the surface of the cells; the product would be released into the medium and subsequently enter the cells where further oxidation could take place. In these experiments, therefore, later products might have been removed from the samples along with the intact cells and other trichloracetic acid-insoluble and heat-coagulable material.

The enzymatic character of nicotine oxidation in these experiments was demonstrated by the fact that samples which were acidified and heated immediately after addition of microorganisms showed no change in the typical nicotine absorption spectrum after 48 hours.

Cell-free protein fractions from the bacillus P-34 have been obtained that catalyze the successive oxidation of nicotine to 6-hydroxynicotine(1), 6-hydroxypseudooxynicotine(2) and 2,6-dihydroxypseudooxynicotine (3). Further oxidation led to production of the unidentified blue pigment(2). The first step in nicotine oxidation by the Arthrobacter species is the same as that of P-34 and the blue pigment is also produced, suggesting that the rest of the pathway might be the same. This possibility is currently being investigated.

The rate of nicotine oxidation was greatest in the Arthrobacter species during the fragmenting stage in the life cycle of the organism. This may reflect the rapid increase in "total cell substance" reported by Sguros(8) for this stage of *Arthrobacter oxydans* growth. It is also possible that the enzymes responsible for catalysis of nicotine oxidation were not elaborated until this stage of maturation was reached.

Maximum concentration of 6-hydroxynicotine detectable in the medium was approximately 50% of initial nicotine concentration. Since the blue pigment appeared to be a nicotine metabolite, its production would account for at least part of the remaining nicotine.

However, the greater share of the remaining nicotine can be accounted for if it is assumed that the 6-hydroxynicotine was taken into the bacterial cells and was further metabolized intracellularly. The extent of conversion of nicotine to 6-hydroxynicotine could then have been greater than 50% if oxidation of nicotine and absorption of the product into the cells were taking place simultaneously.

Summary. A gram negative, non-motile

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‡ Dr. Sydney C. Rittenberg kindly supplied a sample of 6-hydroxynicotine produced by the bacterium, P-34.

§ Performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

bacterium isolated from tobacco plant roots was identified as a member of the genus Arthrobacter. The organism had the ability to convert nicotine to 6-hydroxynicotine. Maximum concentration of 6-hydroxynicotine detectable in the culture medium was approximately 50% of initial nicotine concentration on a molar basis. The identity of the product was established by its melting point, and the melting point of its picrate, its specific rotation, elementary analysis, and ultraviolet and infrared absorption spectra.

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Synergism Between Staphylococci and Proteus in Mixed Infection.[*]

(26881)

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Synergism among bacteria in mixed infection is not a new concept although carefully studied cases of specific synergism *in vivo* appear to be relatively sparse in the literature. Even with the current emphasis on staphylococcal infection few studies on synergism between staphylococci and other organisms have been reported(1-3). It was recently noted in this laboratory that mice receiving mixed infections of *Staphylococcus aureus* and *Proteus vulgaris* died rapidly with fulminant septicemia although neither of these organisms alone appeared to have any deleterious effect on the animals when injected in comparable numbers and by the same routes.

This report presents the preliminary results of an investigation of this phenomenon.

Materials and methods. All mice[†] used in these experiments were young adult Webster-Swiss females weighing from 17 to 20 g. Prior to use in specific experiments the animals were held in large cages in an isolated stock-room. No evidence of spontaneous infectious disease in the stock animals has been observed even when they have been held up to 3 months in these quarters. (These older mice were not used for the reported experiments). The standard diet was Purina rat chow pellets and water supplied *ad lib*. For infection experiments the mice were transferred to steel box-type cages. Inoculation of these animals was carried out in a room separate from the stock-room where the test mice were then held for observation until experiments were terminated.

The staphylococci[‡] used in these observations were isolated from human infections. Both the staphylococci and gram-negative bacteria used were routinely maintained at room temperature on Trypticase-Soy agar slants. Liquid cultures were grown in Trypticase-Soy broth at 37° C under fully aerobic conditions. In all cases the inocula were dilutions of 18 hour cultures and were of remarkably uniform titer regardless of the strain of staphylococci employed. As determined by the usual method of dilution and plate-counting the range of 18 hour titers for the staphylococci was 1.6-2.0 × 10^8, and for the proteus was 1.6-1.8 × 10^9. Any alterations from these standard procedures will be noted.

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[*] Supported by U.S.P.H.S. grant.

[†] Obtained from Dovel Farms, College Park, Md.

[‡] Kindly supplied by Mr. Matthew Fusillo, D. C. General Hospital and Dr. James A. Curtin, Georgetown Univ. Hospital.

Except where indicated, inoculation of the mice was accomplished without anesthesia by intraperitoneal (I.P.) injection using tuberculin syringes and ⅝ inch #22 disposable needles. Intravenous (I.V.) injections were made into the dorsal or lateral tail veins using ⅝ inch #27 needles.

Results. 1. *Original Observations on Synergism.* The initial observations on this phenomenon indicated that there was an unquestionable increase in deaths of those mice inoculated with both staphylococci and proteus over those inoculated with either alone, even when the doses were adjusted so that the total number of organisms injected was the same in mixed infection as in the pure-culture inoculations. It has been stated above that neither staphylococci nor proteus had deleterious effects on the mice, but this is true only when some qualification is placed on the size of the inoculum, since deaths were regularly observed if a sufficient dose of an undiluted proteus culture was injected. The course of disease produced by such injections, however, was entirely different from that observed following the mixed inoculations. The former was much like that seen with a large inoculum of any broth-grown enteric organism and was characterized by marked diarrhea, dehydration, and prostration of the animals beginning within several hours of the injection. Furthermore this same phenomenon could be produced (although to a lesser extent) by injection of sterile culture filtrate. However, the same number of proteus when washed with sterile saline and resuspended at an equivalent titer was not uniformly lethal. From these observations it was concluded that death of the mice following injection of undiluted proteus was due to accumulated toxic products in the broth rather than to inherent pathogenicity of the organisms themselves. In contradistinction, the disease produced by mixed inoculation was slower, did not involve severe diarrhea and terminated with marked septicemia, and death. When autopsied these animals showed invasion of virtually all tissues by the infecting agents, predominantly the proteus. In most cases death occurred within 36 hours after injection although occasionally some mice have survived as long as 48 hours. In the cases where mice have been held longer than 48 hours no additional deaths have been observed for as long as 30 days; thus the standard time for the following observations was 48 hours with all experiments terminated at that point.

Since it is known that gram-negative bacterial endotoxins injected into mice with, or prior to, an experimental bacterial infection appear to enhance the infectious process(4), the initial working hypothesis of the staphylococcus-proteus interaction was that endotoxin from the proteus acted to depress the resistance of the mice to the staphylococci. It shortly became apparent that this was not the case but that the reverse was true. Table I presents supporting data for this conclusion, suggested by the results of using various combinations of live and heat-killed cultures. Also included in this table are observations made with partially purified extracts of staphylococci. These data were obtained using commercially available§ "Staphylococcus lipopolysaccharide," (Difco #0350) although equal success has been obtained using similar materials prepared in this laboratory. These were extracts from "agar-washings" of plate-grown staphylococci prepared by a modification(5) of the Boivin trichloroacetic acid (TCA) procedure for extracting bacterial lipopolysaccharides(6).

The pooled data presented in the first table represent number of deaths per total number of mice tested in a series of experiments. For these studies the dose of proteus was 0.1 ml of a 1:10 dilution of the 18 hour culture, (ca 10^7 bacteria). Both live and killed staphylococci were injected at a dose of 0.2

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§ Difco Laboratories, Detroit, Mich.

TABLE I. Synergistic Effect of *S. aureus* and *P. vulgaris* in Mixed Infection: Mouse Mortality at 48 Hours.

	Live staph. culture (0.2 ml, I.P.)	Heat-killed staph. culture (0.2 ml, I.P.)	Difco staph. extract (200 μg)	Controls (no staph.)
Live proteus culture (1:10 dil'n—0.1 ml)	19/20*	12/20	17/20	0/20
Heat-killed proteus culture (1:10 dil'n—0.1 ml)	1/15	0/15	not tested	0/15
Controls (no proteus)	0/15	0/15	0/15	—

* No. dead/Total No. inj.; scored after 48 hr.

ml of an undiluted 18 hour culture. When "lipopolysaccharides" were substituted for live or heat-killed staphylococci the I.P. dose was 200 μg.

From these data it can be seen that the staphylococcus-proteus combination was lethal for mice only when viable proteus was present, although live or heat-killed staphylococci and their TCA extracts were all capable of acting in combination with the proteus. Thus, the phenomenon seems to represent something other than enhancement of infection by gram-negative endotoxins.

2. *Effect of injection route.* Since the mechanism of the interaction did not appear to be that originally proposed, it was thought that the lipopolysaccharide-containing extract from the staphylococci might conceivably act in a physical way much as hog gastric mucin is believed to enhance the virulence of certain bacteria. The long list of such infection promoting agents includes a large number of high molecular weight materials including some bacterial polysaccharides(7). The most direct way to test the action of the staphylococcal material in this respect seemed simply to deliver it by a different route from the proteus challenge. Consequently experiments were done in which the proteus was injected I.P. but the staphylococci or staphylococcal-extract was injected I.V. For these observations doses of materials chosen were the same as those previously used. The results (Table II), demonstrate that even when injected by an entirely different route from the challenge infection the staphylococcal extracts or staphylococci were capable of potentiating the virulence of the proteus but in themselves had no apparent toxicity.

TABLE II. Effect of Injection Route on *S. aureus P. vulgaris* Interaction in Mice.

	Live staph. culture		Difco staph. extract	
	I.P.*	I.V.*	I.P.*	I.V.*
Proteus culture*	10/10†	3/10	7/10	6/10
Controls (no proteus)	0/10	0/5	0/10	1/5

* Same doses as in Table I.

† No. dead/Total No. inj.; scored after 48 hr.

3. *Effects of other staphylococcal strains.* Since all of the above observations had been confined to a single coagulase-positive strain of *S. aureus,* it was of interest to determine whether this action was a general property of other staphylococci or unique to the strain under observation. Ten coagulase-positive strains including *S. aureus,* phage type 80/81, as well as 5 different coagulase-negative *S. epidermidis* isolates have been tested. Table III includes only the results of 3 each of the *S. aureus* and *S. epidermidis* strains. The results with the rest of the strains were exactly comparable to those presented. While there was some variation in efficacy of the various *S. aureus* strains tested, all showed some effect in this system. On the other hand, none of the *S. epidermidis* strains tested has shown significant effect in enhancing the virulence of the proteus challenge.

4. *Effects of staphylococci on other gram-negative infections.* A previous report has indicated a synergistic effect between staphylococci and pseudomonas in mixed infection (3). It was therefore of interest to observe whether this phenomenon was applicable to other genera of the gram-negative enteric organisms. The preliminary results obtained will be reported elsewhere. It may be noted here that in the small numbers of mice examined other gram-negative bacteria such as

TABLE III. Effect of Other Staphylococcus Strains in Mixed Infection with *Proteus vulgaris*; Mouse Mortality at 48 Hr.

	Coagulase positive			Coagulase negative		
	80/81	11096	11462	11305	10827	7196J
*P. vulgaris**	11/15	13/15	9/15	0/15	0/15	1/15
Controls	0/10	0/10	0/10	0/10	0/10	0/10

* 1:10 dil'n of live culture; 0.1 ml—I.P.

E. coli, A. aerogenes, and *Pseudomonas* seem to share in the phenomenon.

Discussion. On the basis of the above data it seems evident that under certain circumstances, staphylococci can so enhance the virulence of otherwise relatively innocuous microorganisms as to cause rapid death of the host. Less clear, however, is the importance of this phenomenon in natural infection and further investigation of the time relationships and the quantitative aspects of the interaction must be made.

The second important question concerns the mechanism of this event. The effects of gram-negative bacterial endotoxin previously mentioned are well documented in a variety of test infections, and in many ways the staphylococcal extracts used in these studies resemble this closely. Higginbotham and Bass(8) have recently reported that similar extracts of *S. aureus* show other properties of endotoxin such as tumor necrosis and preparation for the dermal Shwartzman reaction. Without further data, however, one cannot equate the staphylococcal material with true "bacterial endotoxin."

Finally, the marked difference between coagulase-positive and coagulase-negative negative staphylococci as to their ability to enhance the virulence of a proteus challenge in mice is worthy of additional mention. The number of different strains tested is not sufficient to generalize about this as a further property of "virulent" or "pathogenic" staphylococci although the correlation has so far been excellent.

Summary. Evidence has been presented indicating that *in vivo* synergism exists between *Staphylococcus aureus* and *Proteus vulgaris* when these organisms are injected into Webster-Swiss mice. By using combinations of live and heat-killed cultures of these organisms as well as extracts of the staphylococcus it has been demonstrated that the apparent lethal infectious agent in this combination is proteus and that some product elaborated by the staphylococcus is responsible for the enhancement of virulence of the proteus challenge. Although the mechanism of this interaction is not understood, it has been suggested that in this phenomenon the staphylococcal product acts in a manner similar to that of gram-negative bacterial endotoxins. Furthermore, this material is extractable by procedures used for extraction of such endotoxins. It has been shown also that the *Staphylococcus epidermidis* strains so far examined do not appear to have the same infection-enhancing properties as the coagulase-positive *S. aureus* strains which have been tested.

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Effect of High Concentrations of Nystatin upon Glycolysis and Cellular Permeability in Yeast.* (26882)

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The polyene antifungal agent nystatin inhibits the ability of fungi, especially yeasts, to grow and to utilize various substrates(1-3). Following treatment of yeast with nystatin, there is a leakage of acids in the tricarboxylic acid cycle(4,5), of inorganic phosphate (2,3) and of K^+(6). Since nystatin is equally inhibitory to glycolysis by intact yeast and by protoplasts, but has no effect on cell-free extracts(7), it was suggested that binding by the cell membrane may be the critical step in nystatin action(6). The resulting membrane damage would result in leakage of metabolites and death of the cell. The importance of permeability alterations in polyene action has also been indicated by Bradley and Jones (2), Drouhet, *et al.*(3), and Kinsky(8).

With low concentrations of nystatin, inhibition of glycolysis is apparently the result of K^+ leakage, since the inhibition at neutral pH can be prevented or reversed by exogenous K^+ or NH_4^+(6). Under these conditions the K^+ loss still occurs and the fungicidal action of the polyene is not prevented. Evidence is now presented that at higher concentrations of the antibiotic, a $K^+ - NH_4^+$ mixture will protect or restore glycolytic activity only in stationary phase cells and here only partially. For log phase cells, all the cofactors that are required in a cell-free glycolytic system must be added. The changes occurring in nystatin-treated cells and protoplasts are described, with particular emphasis on indications of altered membrane permeability.

Materials and methods. Saccharomyces cerevisiae strain LK2G12 was used for the present studies. Log phase and stationary phase cells and protoplasts were prepared as described by Marini *et al.*(6).

A highly purified preparation of nystatin (Lot HV 942, obtained from the Squibb Institute for Med. Research, New Brunswick, N. J.) was dissolved at 15 mg/ml in dimethyl sulfoxide and diluted in water. The citrate-phosphate buffers were those described by Gomori(9). Na^+ was replaced in certain experiments by K^+ or NH_4^+. For manometric measurements, Warburg flasks were flushed with nitrogen and equilibrated at 30°C. The yeast juice was prepared by freezing and thawing bakers yeast(10) and was heated for 5 min in a boiling water bath. The contents of the flasks are described in the text. Abbreviations used: ATP, adenosine triphosphate; AMP, adenosine-5′-phosphate; DPN, diphosphopyridine nucleotide; TPP, thiamine pyrophosphate; NY, nystatin; $(K^+ - NH_4^+)$, a mixture of potassium and ammonium chlorides.

Results. Cofactor requirements of NY-treated cells. When 10 μg/ml or more of NY were added to log phase yeast cells in Na^+-buffer, pH 7.0, glycolysis was inhibited almost immediately (Fig. 1). No protection was afforded by 36 mM KCl and NH_4Cl, although one-fourth of this level prevented inhibition by 5 μg/ml of NY. In the presence of the cofactors required for glycolysis by soluble extracts, including small amounts of pyruvate as an oxidant primer, CO_2 production continued although at a rate only 30 to 40% of that in the absence of NY. With stationary phase cells, protection could be achieved by adding 36 mM $(K^+ - NH_4^+)$ or, in other experiments, by 9 mM $(K^+ - NH_4^+)$ and cofactors.

Similar results were obtained when the reversal of an existing inhibition by high NY concentrations was studied, although in this case both cell types reached only 30 to 40% of the uninhibited rate. High $(K^+ - NH_4^+)$ levels were again effective only for stationary phase cells. The need for all the individual cofactors for reversal was somewhat more

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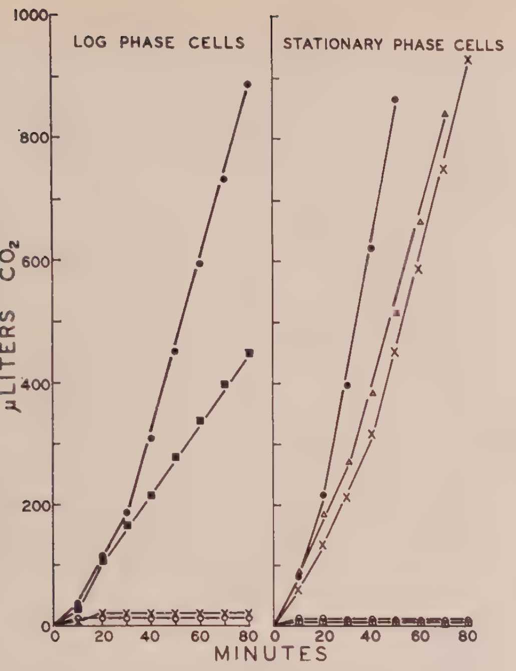

FIG. 1. Protection of glycolysis against high concentrations of NY. Each flask contained 1 × 10^8 cells, 0.1 M sodium citrate phosphate buffer, pH 7.0, 120 μmoles glucose, 180 μg NY, and supplements as indicated in 3.0 ml. Glucose, NY, and supplements added at 0 min. ●, Control; ○, NY; □, NY + 9 mM KCl and NH_4Cl; ×, NY + 36 mM KCl and NH_4Cl; ■, NY + 9 mM KCl and NH_4Cl + cofactors as in Table I; △, NY + 36 mM KCl and NH_4Cl + cofactors.

clearly defined than in protection experiments. Results with log phase cells are presented in Table I. In other tests, omission of ($K^+ - NH_4^+$) prevented reactivation. ATP could be replaced by equimolar AMP; as little as 0.1 μmole of either nucleotide produced about half-maximal reactivation. TPN could not be substituted for DPN. In several experiments, 0.3 to 2.0 ml of the boiled yeast juice was added per cup. Glycolysis by both NY-treated and intact yeast increased ca 20%, but the rate with the treated cells was still only one-third of that observed in absence of NY. Increased cofactor concentrations also failed to affect this relative rate.

TABLE I. Requirements for Glycolysis by Cells Treated with High Levels of NY.

NY, μg/ml	Supplement omitted	μl CO_2 0-25 min.	μl CO_2 25-95 min.
None		205	1100
60		74	470
60	ATP + DPN	63	74
60	Pyruvate	64	55
60	Mg^{++}	38	70
60	TPP	42	65
60	Glucose	4	20

The flasks contained 1 × 10^8 log phase cells in 3.0 ml of .05 M ammonium citrate phosphate pH 7.0 and the following supplements (μmoles), except where indicated: 120 glucose, 20 $MgCl_2$, 10 ATP, 5 DPN, 10 sodium pyruvate adjusted to pH 7.0, 5 TPP, and 27 μM each of NH_4Cl and KCl. Gas phase, N_2; temperature, 30°C. NY and glucose were added from a sidearm at 0 min. After 25 min. when metabolism had ceased, pyruvate was added.

It should be noted that at pH 4.0 neither protection against NY nor reversal was obtained, even with the combination of cofactors and high ($K^+ - NH_4^+$) levels or with the boiled yeast juice. This was anticipated, since a rapid destruction of enzymes occurs following treatment with NY at this pH(5).

Action of NY on protoplasts. The cofactor requirement at high NY levels was also determined for these altered cells. In this experiment (Fig. 2) all supplements except pyruvate were added at 0 min. Glycolysis ceased within 20 min in those vessels containing NY. At 40 min, pyruvate was added and CO_2 production measured. As previously noted with intact cells, the maximum rate attained by treated protoplasts was ca 30% of that in the absence of NY. Omission of any constituent resulted in a sharp reduction of glycolysis.

Lysates of protoplasts did not produce CO_2 from glucose unless the entire complement of cofactors was present (Fig. 2). Under these conditions, NY had no effect on glycolysis even at 90 μg/ml. The maximum glycolytic rate by lysates was 30-40% of that obtained with intact protoplasts.

NY-treated cells or protoplasts showed fermentation rates and cofactor responses essentially identical to those obtained with lysates. They cannot be considered as equivalent to lysates, however, since glycolytic activity was not released into the medium by the treated cells (Fig. 3). Suspensions of 1 × 10^8 protoplasts were incubated at 30°C for 20 min in 1.75 ml of pH 7.0 sodium citrate-phosphate buffer containing 0.82 *M* mannitol and either 0 or 30 μg of NY/ml. The treated proto-

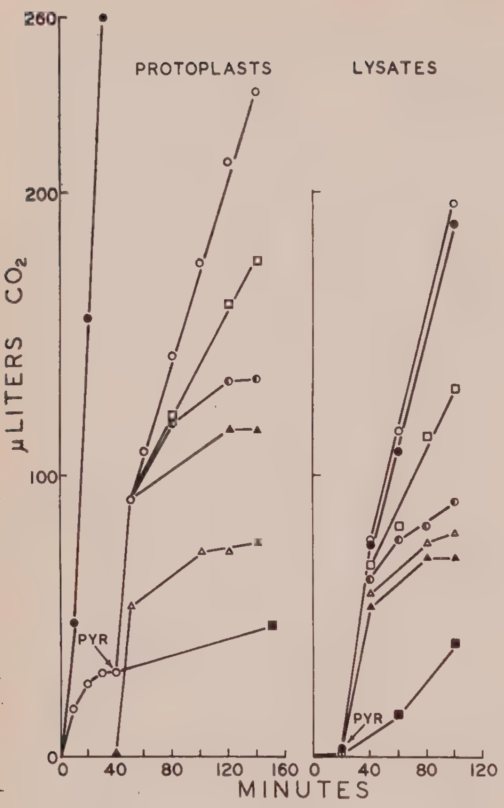

FIG. 2. Glycolysis requirements by NY-treated protoplasts and by lysates. Complete test system: 1.4 × 10^8 protoplasts in 0.04 M sodium citrate phosphate, pH 5.8, containing 0.82 M mannitol (or lysate prepared by suspending 1.4 × 10^8 protoplasts in buffer without mannitol), 2.4 μmoles $MgCl_2$, 7.5 μmoles NH_4Cl, 5 μmoles ATP, 2.5 μmoles DPN, 2.5 μmoles TPP, 30 μmoles glucose, and 5 μmoles pyruvate (pH 7.0) in 2.0 ml volume. ●, Complete system = A; ○, A + 30 μg NY/ml = B; △, B without glucose; ■, B without pyruvate; ▲, B without ATP and DPN; ◐, B without NH_4^+; □, B without TPP.

plasts were collected by centrifugation for 5 min at 1500 × g and resuspended in 1.75 ml of the buffered mannitol. Residual glycolytic activity was then determined in the presence of the following (μmoles): 30 glucose, 5 ATP, 5 DPN, 40 $MgCl_2$, 14 KCl, 14 NH_4Cl, 2.5 TPP, 5 pyruvate, and water to 2.0 ml. With the complete system, the rate of glycolysis by protoplasts preincubated with NY was ca one-fourth that exhibited by those preincubated in its absence—a relationship close to that existing before the washing procedure (Fig. 2). The treated and washed protoplasts were still completely dependent on (K^+ – NH_4^+) and pyruvate and partially dependent on one or more cofactors. No significant glycolytic activity was found in the supernatant fluid from the NY-treated protoplasts. Leakage of an incomplete complement of glycolytic enzymes has not, however, been excluded.

Of considerable interest is the fact that the treated cells still retain permeability barriers to sugar phosphates. Thus, glucose-6-phosphate and fructose diphosphate were not utilized by the treated cells even in the presence of the cofactors and primer required for CO_2 production from glucose. The same preparations were fermented readily by soluble extracts.

Alterations in cells. The relation between K^+ and NH_4^+ loss and the inhibition of glycolysis is illustrated by the data of Table II. At pH 5.8 in the presence of mannitol, gly-

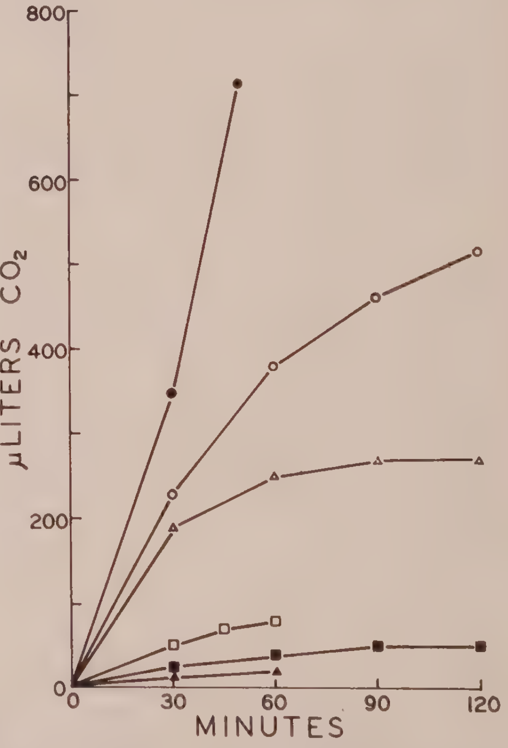

FIG. 3. Glycolysis of protoplasts pre-incubated with NY and then washed. See text for complete test system. NY-treated protoplasts: ○, complete supplement; △, no ATP, DPN, or TPP; □, no (K^+ – NH_4^+); ▲, no pyruvate. ●, no NY during pretreatment (complete supplement). ■, supernatant fluid (complete supplement).

TABLE II. Alterations in NY-Treated Yeast.[*]

			NH_3		Leakage of UV-absorbing material, O.D. (260 mμ) of medium[‡]	Pyruvate decarboxylation[§]	
Buffer	NY, μg/ml	K^+ in cells, μg/mg	In cells, μg/mg	In medium, μg/mg cells[†]		Lag, min.	Rate, μl CO_2/30 min.
pH 5.8 NaC-P + .82 M mannitol	0	15.7	2.1	.3	.100	—	4
	1[‖]	.06	1.3	.87	.100	25	66
	2		—			19	161
	5		.17			10	232
	10		—			6	297
	30		<.05	1.42	.156	2	327
pH 7.0 NaC-P	0	11.1	.90	.0	.015	—	20
	2	5.9	.45	.34	.080	90	38
	6[‖]	.05			.075	40	63
	10				—	22	95
	30		<.1	.73	.205	16	144

[*] Log phase cells used. K^+ and NH_3 and O.D. data represent values following incubation with NY for 30 min. NaC-P = 0.03 M sodium citrate-phosphate buffer.

[†] Corrected for NH_3 formed from NY during the analytical procedure (12.3 μg/mg—see text).

[‡] Corrected for O.D. at 0 min. of mixtures without NY (.300 in pH 5.8 NaC-P + mannitol; .245 in pH 7.0 NaC-P).

[§] NY added to 10^8 cells and 200 μmoles pyruvate in 3 ml of buffer. The lag period before decarboxylation and the subsequent constant rate of CO_2 evolution are presented.

[‖] Rapid and complete inhibition of glycolysis occurred.

colysis of log phase cells is inhibited almost at once by 1 μg of NY per ml. At pH 7.0 without mannitol, cells are less sensitive, ca 5 μg per ml being required for rapid inhibition. This difference in sensitivity is attributable primarily to the high mannitol concentration (6), which may accelerate leakage of ions from a partially damaged cell membrane. Similarly, almost complete loss of K^+ occurred with 1 μg of NY at acid pH, whereas 6 μg per ml were required at pH 7.0. Cellular NH_4^+ was also lost following exposure to NY but the concentrations of the antibiotic required were generally higher than those needed to initiate K^+ leakage.

For measurement of ion loss, approximately 6 mg of log phase cells were incubated with the indicated levels of nystatin in 12 ml of buffer containing 0.04 *M* glucose. The suspensions were shaken 30 min at 28°C and then centrifuged. Certain supernatants were examined for leakage of ultra-violet absorbing material, or analyzed for NH_3 by the Markham technic(11). The treated cells were suspended in 5 ml water and placed in boiling water bath for 5 min. The cells were removed by centrifugation, and the extracts analyzed for K^+(6) or for NH_3(12). Levels of NH_3 in the original supernatants were too low to permit estimation by the specific Conway technic. Relatively good recoveries of the NH_3 lost from the cells were obtained by the Markham procedure when correction was made for NH_3 arising from NY during alkaline distillation.

Although the intact yeast cell is apparently impermeable to the pyruvate ion and does not decarboxylate pyruvate at neutral pH, damage of the membrane by freezing (10), drying(13), or by surfactants(14), will permit access of the ion to the decarboxylase. Yeast treated with NY will also decarboxylate pyruvate (Table II). Several points are of special interest: 1) Lower NY concentrations are required to initiate decarboxylation in pH 5.8 buffer containing mannitol than in pH 7.0 buffer, in parallel with the relative sensitivities of glycolysis to NY. 2) Decarboxylation of pyruvate occurs only after a lag and at concentrations of NY considerably higher than those required to produce K^+ and NH_4^+ loss and inhibit glycolysis. Thus, permeability to pyruvate appears to be a secondary result of the initial injury to the cell membrane. 3) Constant rates of CO_2 production were observed following the lags, and the rate was dependent on NY concentration. This suggests an all-or-none acquisition of permeability to pyruvate by the yeast, as Scharff and Maupin(14) noted with benzalkonium chloride. 4) In pH 4.0 buffer, addition of 10-30 μg of NY per ml produced a rapid destruction of pyruvate decarboxylase comparable to that noted by Scholz, *et al.* (5) for other glycolytic enzymes. At pH values intermediate between 4 and 5.8, both an increased accessibility of the decarboxylase and gradual inactivation could be demonstrated.

In parallel tests, there was no pyruvate decarboxylation or K^+ leakage with the highest levels of solvent (dimethyl sulfoxide) used in any of these experiments. Also, the methyl ester and N-acetate of NY, which do not inhibit growth, did not initiate decarboxylation or leakage even at 30 μg per ml.

A detectable loss of ultra-violet-absorbing substances (acid-soluble) occurred even in the absence of NY (Table II) confirming the report by Higuchi and Uemura(15). This loss increased in the presence of NY, especially at those high concentrations which produced a deficiency of the nucleotide cofactors essential to glycolysis. At 30 μg NY per ml, some loss of protein from the cell was observed in several, but not all, experiments.

Discussion. A graded series of effects upon yeast permeability can be obtained by addition of increasing concentrations of nystatin. The pH range of 5.8 to 7.0 was used to avoid secondary autolytic changes believed to occur at acid pH(1). At the lowest levels of nystatin, the only alteration observed thus far is the rapid loss of K^+ ions(6), which correlates well with inhibition of glycolysis. NH_4^+ ions, which can also restore glycolytic activity when added to the system, are not eliminated from the cell unless higher nystatin levels are used. However, the concentration of ammonia in the cell is low, and values obtained even with the comparatively mild Conway procedure may still represent substances other than ammonium ion available for glycolytic activity. The results are consistent, therefore, with the idea that K^+ depletion is the critical factor in the inhibition. Although in the test system used by Marini *et al.*(6) no effect of phosphate ion could be shown, in pH 7 sodium acetate buffer under the general conditions of Bradley and Farber(16), both phosphate ions and K^+ or NH_4^+ were required for reversal of nystatin inhibition of glycolysis. Thus, phosphate leakage must also take place at relatively low nystatin concentrations. Increasing the level of nystatin will initiate leakage of UV-absorbing nucleotides and cause sufficient change in the membrane that decarboxylation of the pyruvate ion is possible. Leakage of pyruvate, malate and other carboxylic acids and subsequently of sugar phosphates occurs under approximately the same conditions(4,5). This leakage, and consequent dilution of cofactors and of intermediates, especially pyruvate, is probably the reason glycolysis halts under these conditions. At the highest nystatin levels, there is some loss of protein, but the bulk of the glycolytic enzymes must remain with the cell.

At least partial reactivation of CO_2 production can be attained by addition of external glycolytic cofactors. It should be noted that this response to external cofactors can be demonstrated only with very high nystatin levels and with the most sensitive cell types, that is, log phase cells or protoplasts. The reversal experiments presented here emphasize the role of pyruvate in initiation of glycolytic activity but, as seen from the experiments on protection, the other cofactors are also required under comparable conditions. A requirement of external ATP for glycolysis has also been reported recently by Fox and Stevenson(17) for streptococci whose permeability had been altered by treatment with trypsin.

Even at the highest nystatin levels the yeast membrane does not appear to be open to the entrance of all ions by simple diffusion. For instance, the cell retains the barrier to glucose-6-phosphate and fructose diphosphate characteristic of yeast. The cellular alterations at lower concentrations of the antibiotic are probably even more specific, particularly since there is significant loss of only certain cell constituents.

Summary. Permeability alterations induced in yeast by high levels of the polyene antifungal antibiotic nystatin were examined. Glycolytic activity could be protected

against nystatin either by adding ca 0.04 *M* K^+ and NH_4^+ salts (stationary phase cells) or by 0.01 *M* K^+ and NH_4^+ salts and the cofactors essential for a cell-free glycolytic system (log phase cells or protoplasts). Under these conditions there was leakage of K^+, NH_4^+, nucleotides and some protein, although glycolytic activity remained with the treated cells or protoplasts even after repeated washing. Pyruvate decarboxylase in NY-treated cells became accessible to pyruvate ions, but glucose-6-phosphate and fructose-1,6-diphosphate were still not fermented. The rate of CO_2 production by NY-treated cells or osmotically-lysed protoplasts did not exceed 25 to 30% of that obtained with the original preparations. The nature of the membrane alterations is discussed.

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Apparatus for Change of Medium in Extracellular Maintenance *in vitro* of an Intracellular Parasite (Malaria). (26883)

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Methods have been described whereby the avian malarial parasite, *Plasmodium lophurae*, can be removed from its host erythrocytes and maintained extracellularly *in vitro* for up to 4 days at 40°C(1,2). These methods have made possible a study of some of the nutritional requirements of an intracellular parasite itself, as distinguished from the host cell-parasite complex.

To get the best survival and development it was necessary to change the culture fluid after the first 18 hours and at 12 hour intervals thereafter. This was done as follows: Each 50 ml Erlenmeyer culture flask received 0.25 ml of duckling plasma (obtained without use of an anticoagulant). To this was added 0.02 ml of a chick embryo extract (1 10-day embryo ground in 4 ml of glucose-salt solution, mixture centrifuged, supernatant used). The flask was then rotated rapidly in such a way that the plasma clotted as a thin layer along the lower part of the wall of the flask, with the excess sometimes forming a larger clot at the bottom. Each flask then received the culture medium and finally the inoculum of hemolyzed red cells and free parasites prepared by the treatment of blood from an infected duckling with guinea pig

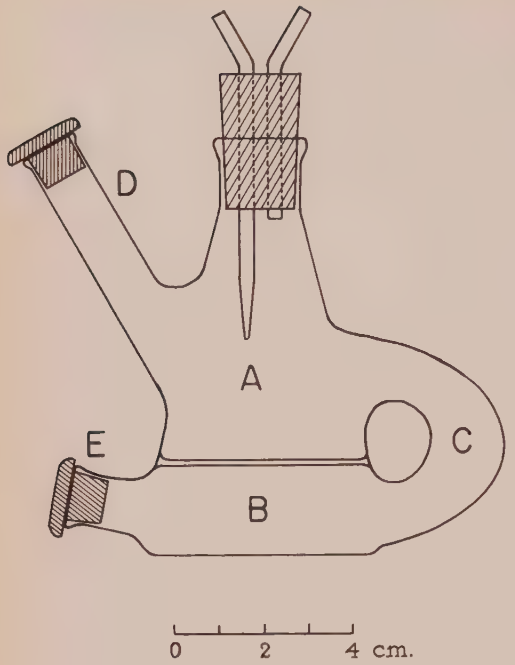

FIG. 1. Diagram of special culture flask with two connected compartments.

serum (as complement) and an anti-duck erythrocyte serum prepared in rabbits. The flasks were placed at 40°C on a rocking device giving 16 complete cycles per minute and a tilt of 20° from the horizontal. The cultures were usually completed at about 4 p.m. (of day 0). By the morning of day 1 approximately half of the free parasite-hemolyzed red cell material had stuck to the layer of plasma clot to form a scum which was washed over by the culture medium as the flask rocked to and fro.

The scum formation made it a relatively simple matter to draw off the fluid in the flask with a pipette and replace it quickly with an appropriate volume of fresh, previously warmed medium. All attempts to separate parasites from old culture fluid by centrifugation and resuspension in fresh medium resulted in damage to the organisms. The change of medium was effected after the first 18 hours (at 9-10 a.m. of day 1) and thereafter at approximately 12 hour intervals.

When survival to the 4th day was being regularly obtained, it became necessary to take care of the cultures in any one experiment on 3 successive nights, a procedure which made difficult any still more prolonged experiment. The following apparatus was accordingly devised.

Culture flask. This was a 50 ml Erlenmeyer flask provided with a compartment (B) below it connected to the main body of the flask (A) by a U-shaped arm (C) (Fig. 1). Opposite this U-shaped arm a long straight side-arm (D) extended from the body of the flask and a short side arm (E) from compartment B. The side arms C and D left the flask at such a height that when 4 ml of medium was rocked to an angle of 20° it did not overflow into C. Arm C purposely had an expanded diameter at the base of the U. If the flask was tipped in the direction of arm C through an arc of over 100° the fluid in A entered arm C and then entered compartment B when the flask was righted. Arms D and E were made to fit red rubber vaccine stoppers. Compartment A was stopped with a silicone rubber stopper bearing delivery tubes for the passage of a slow current of air with 5% CO_2 through the flask.

The changing apparatus. Fig. 2 gives an overall view of this with the doors open. It consisted of 2 main parts—an incubator(1) for the culture flasks and a refrigerator(2) for storage of the fresh medium. Part 1 contained a heating unit, thermostat and a rocking platform equipped to hold 8 of the culture flasks. The rocking was effected by a motor driving an eccenter (No. 1, Fig. 3) which transmitted the motion by a steel band. A switch could be set so that at a desired time a time-clock activated a solenoid which engaged eccenter No. 2. This moved the platform through an arc of 115° from the horizontal (Fig. 3), thereby tipping the flasks in the direction of arm C (Fig. 1) and emptying the culture compartment A. Eccenter No. 2 went through only one revolution before being disengaged by the solenoid, the motion of the platform then returning to eccenter No. 1. At the same time solenoids in the upper unit 2 (Fig. 2) were activated by a relay to open clamps which had been shut on silicone rubber tubing connecting a reservoir tube (Fig. 2(3)) to each culture flask. The opening of the clamps permitted fresh

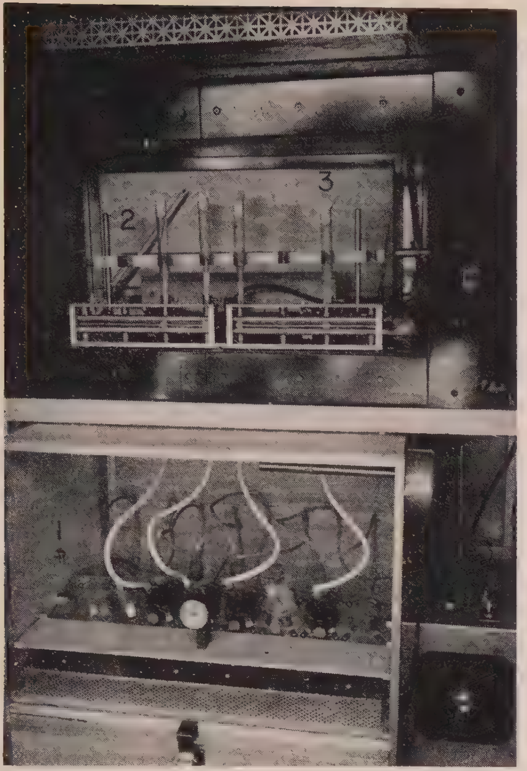

FIG. 2. General view of apparatus (with doors open) just after a change of medium. Unit 2 is supported on a frame over unit 1.

medium to flow into compartment A through side-arm D, after the former had been emptied of the old medium. The solenoids in unit 2 could also be opened and shut by a manually operated switch. Unit 2 was a well insulated refrigerator with a compressor placed above it. It could maintain a temperature of 1-2°C. It contained the 2 clamps activated by the solenoids, each controlling 4 reservoir tubes held in a rack. It was equipped with a time clock and a small heating unit controlled for a temperature of about 40°C. In this way fresh medium in the reservoir tubes was kept cold until 45 minutes before the time of changing of the medium, when the refrigerator was automatically switched off and the heater on. By the time the change occurred the fresh medium had been warmed.

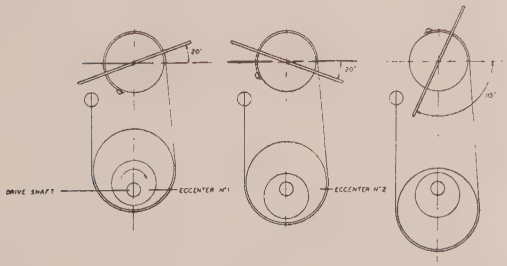

FIG. 3. Diagram illustrating principle whereby rocking motion is changed to a single tipping movement which removes the fluid from the culture chamber.

Use of the apparatus in a culture experiment. Culture flasks were prepared using compartment A (Fig. 1) in the same way as an ordinary Erlenmeyer flask. At this time arms D and E were stopped with the vaccine stoppers. Care was exercised not to tip the completed flasks more than through a small angle in the direction of arm C. The flasks were placed on the rocking platform and the delivery tubes in the central stopper connected to the source of air with 5% CO_2. The incubator had been previously adjusted to 40-40.5°C, this temperature being read from a small thermometer inserted in the side-arm of an empty flask (Fig. 2). On the following morning, when the scum containing the free parasites had formed, the medium was changed manually in each flask. Each flask was removed individually, the previously mixed medium for the flask was taken up in a 5 ml pipette, the flask was opened at the central stopper and rotated so that the old medium contained in compartment A spilled through C into B, the fresh medium being then immediately added.

For the automatic change, to be effected at night, the fresh medium was mixed in reservoir tubes. These were made of Pyrex glass tubing with an outer diameter of 16 mm and about 12 cm long. The lower end was drawn to an outer diameter of about 7 mm and bore a 25 cm length of silicone rubber tubing (4 mm inside diameter) having at the opposite end a 5-6 cm length of glass tubing of 5 mm outer diameter. The free end of this tube was covered with aluminum foil. The wide end of the reservoir tube bore a gauze and cotton plug. These tubes were sterilized by autoclaving. A small screw clamp was attached and closed at the base of the reservoir tube, and the latter was provided with the fresh medium. The clamps in upper unit 2 (previously refrigerated) were opened and the foil-capped free end of the tubing was threaded through the clamps and through a small opening which guided it from the bottom of unit 2 into the top of unit 1. With aseptic precautions the aluminum foil cap was removed and a 35 cm long piece of silicone rubber tubing was attached. This bore at its free end a short length of 7 mm glass tubing surrounded at its upper portion by a 2 cm length of silicone tubing. This fitted snugly into arm D of the culture flask, replacing the vaccine stopper, and completed the connection from the reservoir tube to the flask. The clamps in unit 2 were now shut and the screw clamps at the base of the reservoir tubes were opened. The refrigerator was set to change to the heater at 9:15 p.m. and the change of medium was set for 10 p.m. The following morning each connecting tube was removed and replaced with a vaccine stopper in arm D, sterile precautions being observed throughout.

Since the flasks so far used have a lower compartment (B, Fig. 1) able to hold fluid from only 2 changes of medium, this compartment was emptied once each day. A sterile needle was inserted through the vaccine stopper into arm E and the fluid drawn off with a syringe.

Discussion. With the same medium used previously (Trager(2)), extracellular survival and development of *P. lophurae* have been as good in the automatic changer as in experiments in which the medium was changed by pipetting. On day 4 about 80% of the parasites appeared in good connection by morphological criteria. Recently, a modification of the medium has given good survival in the automatic changer to day 5, with infective parasites demonstrated on day 6.

The apparatus here described is not designed for the same purpose as apparatus providing a continuous slow flow of medium. Apparatus of the latter type would not be suitable since the medium used at present for extracellular maintenance of malaria parasites contains labile ingredients, such as adenosinetriphosphate and, in addition, the concentrated red cell extract contains hydrolytic enzymes. Medium held warm in a reservoir providing a slow flow would deteriorate as much as the medium in the culture vessel. With the present method of sudden change, however, the fresh medium can be refrigerated until shortly before it is added to the culture flask. It then provides at the outset high concentrations of labile factors, which presumably are much reduced by the end of the 12-hour period between changes of medium.

Summary. A special flask and mechanical equipment are described which provide for automatic removal at a desired time of old culture fluid and addition of fresh medium to cultures in which extracellular malaria parasites (*P. lophurae*) are attached as a scum to a thin plasma clot lining the wall of the flask.

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Species Dependence of Chlorpromazine Metabolism.* (26884)

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Earlier reports from this laboratory(1,2) have confirmed the importance of sulfoxidation as a pathway for the metabolism of chlorpromazine(3,4). However, the question

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has been raised whether chlorpromazine sulfoxide (CPSO)† itself is a significant urinary excretion product(1,2). Detailed chromatographic studies(1,2) on human urine collected after oral chlorpromazine therapy revealed only minor amounts of CPSO; in contrast, 2 major unconjugated metabolites were detected whose properties were indistinguishable from the desmethyl sulfoxides (Nor_2 CPSO and Nor_1CPSO). These findings were somewhat unexpected, since CPSO has achieved much prominence in the literature (3,4,5), whereas the nor_2sulfoxide had not been previously reported.

The purpose of the present work was 2-fold: (a) To provide additional confirmatory evidence for the desmethyl sulfoxides; and (b) To determine the basis for the divergent findings between our group and other workers. Towards this end, a comparison has been made between chlorpromazine metabolism in man and animals. The dog was selected for the study because much of the literature is based on this animal without sufficient evidence that the findings are applicable to humans.

Experimental. Urine specimens. Twenty-four hour urine collections were contributed by Hillside Hospital patients of both sexes who were receiving 600 mg chlorpromazine hydrochloride orally per day. Creatinine determinations as well as replicate analyses were carried out to assure true 24-hour specimens. For the animal studies, 4 female mongrel dogs received 8 mg of chlorpromazine hydrochloride/kg/day in oral form. The urine was collected daily for 5 days and kept under refrigeration to minimize decomposition. Control urines were obtained from patients and animals who were not on medication.

Chromatography. The urinary chlorpromazine metabolites were resolved into a non-polar fraction (*A*) and 2 polar (phenolic) fractions, designated *B* and *C*. Fraction *A* was extracted from urine at pH 11.5 with 3 volumes of ethylene dichloride‡ and prepared for chromatography essentially as described previously(1). The residual urine was extracted at pH 9 (Fraction *B*) and again at pH 9 (Fraction *C*) after it had been incubated overnight with one-tenth volume of β-glucuronidase (ketodase, 5,000 units/ml) at pH 4.5. *B* and *C* were chromatographed with the following solvent systems: BzA, EdBzF, n-butanol-ethanol-water (15:2:5) and ammonia (58%)-methanol (1:1). The last system employed Whatman #3 paper that had been previously dipped in acetone containing 10% peanut oil.

Quantitative Analyses. Three methods were used to quantitate the chromatograms, the particular choice being dependent on the relative amounts of metabolites present as well as their proximity to each other. Trace metabolites were stained with persulfate reagent and promptly estimated by visual comparison with a series of graded standards. Derivatives present in substantial or major amounts were stained with Dragendorff reagent and quantitated by means of a recording densitometer (Spinco Analytrol Model RB, B-5 cam) at 500 mμ, slit width 4 mm. When the chromatographic spots were sufficiently discrete from one another, it was possible to employ an alternative procedure as follows. The corresponding areas on unstained duplicate chromatograms were cut out, then eluted by shaking mechanically with 2 portions of methanol. The eluates were filtered and taken to dryness. To the residues were added 0.1 ml water, 0.2 ml of 0.5% methyl orange, 0.2 ml saturated boric acid and 5 ml benzene-isoamyl alcohol (100:5, v/v). The tubes were shaken, centrifuged, and the upper organic extracts containing the dye-amine complexes were read *vs.* a reagent blank at 430 mμ. Metabolite readings were corrected by deducting the significant absorbances given by control urine specimens which were chromatographed along with the test urine samples.

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† The following abbreviations are used: CP = chlorpromazine, CPSO = chlorpromazine sulfoxide, nor_1 = desmonomethyl, nor_2 = desdimethyl, P = promazine, BzA = benzene-acetic acid-water (2:2:1), EdBzF = ethylene dichloride-benzene-formic acid-water (3:1:4:2), and BuAmA = butanol-ammonium acetate (1 M, pH 7)(2:1).

‡ Eastman reagent grade used. Spurious results were obtained with 2 other commercial preparations. (This could be prevented by redistilling to remove a yellow-colored residue).

TABLE I. Chromatographic Evidence for Nor_2-CPSO and Nor_1CPSO Excretion in Human Urine.

	R_f before and after acetylation			
	BzA		BuAmA	
Compound	Before	After	Before	After
Nor_2CPSO	.17	.77	.68	.82
#2	.16	.77	.68	.81
Nor_1CPSO	.24	.79	.76	
#3	.25	.78	.76	

Small corrections were also applied to the standards, based on the readings obtained by eluting the appropriate areas on "blank" chromatograms.

Acetylation Technic. Urine metabolites #2 and #3 were isolated (elution with methanol) from replicate chromatograms in sufficient amount to yield approximately 0.5 mg of each compound. The solutions were taken to dryness and the residues redissolved in 1 ml of 0.01 N HCl. Nor_2CPSO and Nor_1CPSO served as reference standards. They were dissolved as their hydrochlorides in 1 ml water. The solutions were chilled. To each were added 0.1 ml acetic anhydride and 0.2 g sodium bicarbonate with stirring. All mixtures were clear after 25 minutes. The solutions were mixed with 20 ml ethylene dichloride and 0.2 ml cold 1 N NaOH, then shaken and centrifuged. The organic extracts were washed with a small amount of water and dried over sodium sulfate. They were then reduced in volume to 0.5 ml for chromatographic evaluation.

Results. Evidence for Nor_2CPSO and Nor_1CPSO. Reference was made earlier(1) to 2 major metabolites of chlorpromazine (#2 and #3) which are resolved by prolonged chromatography in EdBzF. From their UV spectra, color reactions and chromatographic behavior it was suggested that these compounds are the desmethyl sulfoxides, Nor_2CPSO and Nor_1CPSO. Additional confirmatory evidence is presented in Table I. The indicated compounds were isolated from urine and acetylated as described in *Experimental,* above. The resultant acetyl derivatives were chromatographed in the BzA and BuAmA solvent systems. Their R_f's proved identical to authentic acetyl Nor_2CPSO and acetyl Nor_1CPSO, respectively. These data supplement our observations that compound #2 = Nor_2CPSO and #3 = Nor_1CPSO as adduced by co-chromatography in 3 systems, *viz.,* EdBzF, BzA and BuAmA.

Species Studies. A comparison between man and dog with respect to their metabolism of chlorpromazine revealed some similarities as well as striking differences. Following their placement on oral chlorpromazine medication, dogs void 9 phenothiazine derivatives in Fraction *A* which are chromatographically similar to the 9 compounds previously detected in human urine (ref. 1, Table I).§ Four of these compounds have been identified (#2, #3, #3′ and #6). In addition, dogs excrete trace amounts of a tenth compound (designated #6′) in Fraction *A* which has not been found in significant quantities in human urine. This derivative was detected by eluting spot #6 from the BzA chromatogram with methanol and rechromatographing it with ammonia-methanol (1:1). Two spots now appeared, the major one at R_f 0.03 (chlorpromazine) and a minor one at R_f 0.13. The minor constituent reacted as a secondary amine (positive nitroprusside, weak ninhydrin) and was chromatographically identical to Nor_1CP. There was no evidence for Nor_2CP, whose R_f in this solvent system is 0.25.

Fraction *A* metabolites #1, #5, #7, #8 and #9 have not been identified. With the exception of #5, these compounds occur in minor amounts in pH 11.5 extracts. However, there is some question whether #1 and #9 are actually minor metabolites. They differ from the other Fraction *A* components by yielding a lavender, rather than pink, persulfate test, and correspond in R_f to 2 components of Fraction *B*. This evidence suggests that they are present in *A* as "contaminants." Further

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§ Reference was made in this earlier paper to a tenth compound (compound #4) which appeared as an artifact in the urine of smokers. This material has been identified as cotinine, a metabolite of nicotine(6). Nicotine itself also appears in lesser amount in the urine of smokers at an R_f comparable to metabolite #1, but neither nicotine nor cotinine yields a positive persulfate test. As a consequence, they can be differentiated from the phenothiazine derivatives.

TABLE II. Quantitative Findings by Various Workers (Expressed as Average % of Administered Drug).

	Chlorpromazine				Promazine
	Present workers*		Salzman and Brodie		Walkenstein and Seifter
Urinary product	(man)†	(dog)	(man)	(dog)	(dog)
Nor$_2$sulfoxide	3.7 ± .6	1.1 ± .1			
Nor$_1$sulfoxide	1.8 ± .5	5.1 ± .8			3
Sulfoxide	.4 ± .08	5.1 ± .7	5	12	4
CP or P	.2 ± .06	2.8 ± .6	—	1.3	2.5
Subtotal	6.1	14.1			
Probable total, Fraction *A*	7.5	17.			

* Values represent mean ± stand. dev.

† Male patients, avg wt 75 kg. Analyses based on four 24-hr urine specimens.

consideration of these compounds will be given in a subsequent paper. Compound #5 (mentioned above) appears to be a sulfide whose UV spectrum is similar to chlorpromazine. Its color reactions provide no evidence for demethylation or for the presence of a nuclear oxygen atom. We suspect that the compound is chlorpromazine-N-oxide, but definitive proof of this is not presently available.

Quantitative excretion values for CP, CPSO, Nor$_1$CPSO and Nor$_2$CPSO are summarized in Table II. It is clear that in neither man nor dog is CPSO the predominant sulfoxide. In fact, CPSO is a *minor* metabolite in man and is voided to the extent of less than 0.5% of the administered CP. The major human sulfoxide is the desdimethyl derivative, Nor$_2$CPSO (3.7%), with the monomethyl compound (Nor$_1$CPSO) following in the order of 1.8%. CP appears in trace quantities, 0.2%. Dogs excrete substantial and equal amounts of CPSO and Nor$_1$CPSO (5.1%), while their output of Nor$_2$CPSO is only one-fifth as great but still exceeds the CPSO excretion in man. They void, on the average, more unaltered CP than man. These results indicate a species difference for the nonpolar fractions of man and dog with respect to the sulfoxides as well as the unaltered CP.

Man excretes less than one-half as much Fraction *A* material as the dog (Table II), but he compensates for this by producing a greater amount and variety of phenolic metabolites (Fractions *B* and *C*). The metabolic patterns are complex and cannot be considered here in detail. However, it is pertinent to note that there is a *striking qualitative difference* between the 2 patterns. Human urine contains 2 series of phenolic derivatives, one reacting blue and the other lavender to the persulfate reagent. *The blue series appears to be entirely absent from dog urine.* The relative poverty of phenolic material in the urine of dogs receiving CP medication probably accounts for our observation that such urine gives a weak test with the ferric chloride reagent(7), in contrast to urine from humans on an equivalent drug regimen.

Discussion. Our animal excretion values for CP, CPSO and Nor$_1$CPSO can be compared in Table II with the values for the analogous members of the promazine family which were reported by Walkenstein and Seifter. Good agreement is noted despite the many differences in experimental procedure between the 2 laboratories. The Salzman and Brodie data are also in reasonable agreement with respect to the excretion of CP by man and dog, but differ as to the CPSO output. These authors based their assay on a method (UV spectrophotometry) which also reflects other sulfoxides present in their extracts. This interpretation is borne out by comparing their "CPSO" values to total sulfoxide output (CPSO + Nor$_1$CPSO + Nor$_2$CPSO) calculated from our own data. The totals obtained for man (5.9%) and dog (11.3%) agree quite well with Salzman and Brodie's values of 5% and 12%, respectively. With regard to Nor$_2$CPSO, direct reference values are not presently available. It is curious that this compound—the major human nonpolar metabolite—was not discovered until 1959 (8). According to our data, the ratio of Nor_2CPSO to CPSO is about 9:1 in man and 1:5 in the dog. Based on this consideration, the nor_2sulfoxide might easily be overlooked in animal urine, but not in human urine. It seems likely that earlier studies on human urine were misinterpreted due to the use of chromatographic and spectrophotometric methods which do not differentiate CPSO from the desmethyl sulfoxides.

The 9 compounds present in Fraction *A* of human urine comprise only 7 or 8% of the drug dose. Of this sum, about 5.5% is attributable to the major metabolites in the fraction (Nor_2CPSO and Nor_1CPSO). As a result there is a balance of barely 2% for CP, CPSO and the 5 remaining metabolites. The significance of this may be appreciated by comparing the combined excretion of CP and CPSO in humans to dogs. The value estimated for man, 0.6%, is seen to be less than one-tenth the output of dogs (7.9%) (Table II). The calculation throws into sharp perspective the species dependence of chlorpromazine metabolism, and accounts in part for the apparent divergence between our earlier findings and that of other workers.

Chlorpromazine metabolism is unquestionably complex. The basis for the extensive proliferation of its metabolites lies in the biological reactivity of the chlorpromazine molecule. It appears to undergo hydroxylation with facility(9), giving rise to the compounds in Fraction *B* as well as to the glucuronic acid conjugates of the *B* components (Fraction *C*). The composition of these fractions is presumably related to the number of hydroxyl groups and their disposition in the chlorpromazine nucleus. Additional factors involved in the composition of all 3 fractions (*A*, *B* and *C*) include: susceptibility to sulfoxidation, to mono- or didemethylation, and to other as yet unidentified alterations in the nucleus and side chain. The total number of chlorpromazine metabolites was originally estimated to be 16 or more(2). Our present estimate lies closer to 24, with the final tally subject to revision as our chromatographic techniques are improved.

Summary. Man and dog are compared with reference to their metabolism of chlorpromazine following its oral administration (8 mg/kg). Both qualitative and quantitative differences are noted in the urinary metabolite patterns. Humans tend to favor the excretion of polar derivatives, along with one or 2 major nonpolar metabolites (Nor_2CPSO, 3.7% and Nor_1CPSO, 1.8%). Their output of CP (0.2%) and CPSO (0.4%) is trivial. Dogs excrete less polar material, less Nor_2CPSO (1.1%), more Nor_1CPSO (5.1%) and substantial amounts of CP (2.8%) and CPSO (5.1%). The human polar fractions contain persulfate-blue as well as persulfate-lavender staining metabolites; the blue series is conspicuously absent from dog urine.

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Transient Increase in Resistance of Mice to Experimental Infection Following a Small Dose of X-Radiation.* (26885)

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The literature on the effects of total body exposure to ionizing radiation deals largely with its deleterious effects. A few publications, however, have reported that previous exposure to a small dose of radiation may reduce the 30-day mortality from a mid-lethal dose of similar radiation administered a few weeks later. This phenomenon has been designated "acquired radio-resistance."

Dacquisto(1) has included an excellent critical review of the literature in his report of his observation that the LD_{50} of adult female mice exposed to whole body X-radiation was increased from 487 r to 617 r by a "conditioning dose" of 50 r given 17 days previously. Hagen and Simmons(2) showed that the LD_{50} of rats was raised by 50 r if they had been exposed to 300 r 3 weeks (but not 1 or 2 weeks) earlier, and Sacher(3) has recently found that lower mortalities resulted from a mid-lethal dose of γ radiation among mice conditioned by previous exposure to .6 r per day for 2 or 3 months.

Acquired radio-resistance has not been adequately explained. It seemed possible that one effect of a "conditioning dose" of radiation might be a temporary increase in resistance to bacterial infection which is an important cause of death in mice during the first few weeks following exposure to mid-lethal doses of radiation(4,5). To test this supposition, mice were irradiated with 50, 75, or 100 r (approximately 1/10, 1/7, and 1/5 their $LD_{50}/_{30}$) and challenged at intervals thereafter by intraperitoneal inoculation with graded doses of *Pseudomonas aeruginosa,* one of the enteric microorganisms which not infrequently causes fatal bacteremia in irradiated mice.

Mice. CF-1 female mice, 9-10 weeks old were kept under observation for at least a week before use. Rockland mouse pellets were available at all times. Tap water was supplied in sterile bottles which were changed every day. Cages were changed and autoclaved once a week. All mice in each experiment, irradiated and controls, came from the same shipment.

Irradiation.[‡] The mice were irradiated with a 250 kv, 30 ma maxitron 250 (General Electric) machine using 0.25 mm copper and 1 mm aluminum filters at a target distance of 79 cm and a dose rate of approximately 69 r/min. For details of method of exposure, see Hammond, Ruml, Cooper, and Miller(6).

The test microorganism was a streptomycin resistant strain of *Pseudomonas aeruginosa* used for a number of years in this laboratory to determine the effect of irradiation on susceptibility to bacterial infection(7,8,9,10). This strain was chosen because the infection it produces is rapidly fatal in susceptible animals; *i.e.,* inoculated mice either die within 18-36 hours or survive the observation period of 30 days. Virulence has been maintained by weekly passage in CF-1 mice. An 18-hour culture was washed off an agar plate in 5 ml saline, and any clumps of bacteria were dispersed by shaking in an Erlenmeyer flask containing a few glass beads on an electric rotator for 15 minutes. The suspension was then diluted to provide the desired inocula. The actual numbers of bacteria inoculated were determined from quadruplicate platings of appropriate dilutions of the suspension used. Three groups of 10 irradiated mice and an equal number of unirradiated controls from the same shipment, were inoculated intraperitoneally with approximately 2.0, 1.2 and 1.0 × 10^8 *Ps. aeruginosa.* Before inoculation, the mice were randomized to separate cage mates.

Results. From the resulting mortalities (30

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[‡] Mice were irradiated at the Argonne Cancer Research Hospital, operated by Univ. of Chicago for U. S. Atomic Energy Commission.

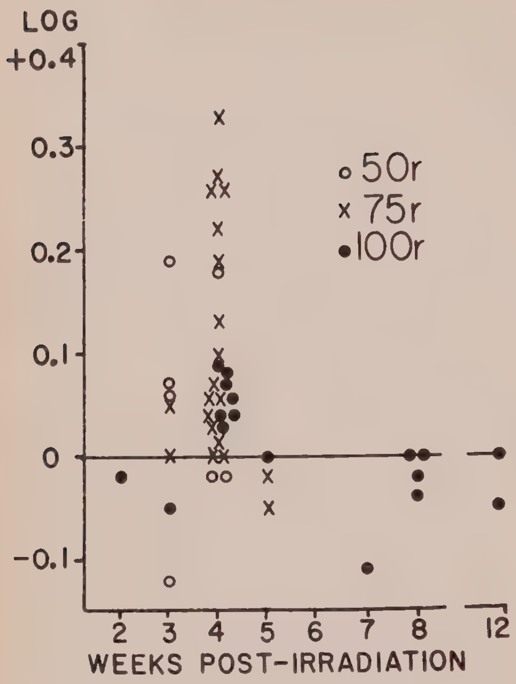

FIG. 1. Increase in log LD_{50} of challenge microorganism for irradiated mice compared with their unirradiated controls.

days) log LD_{50}'s were determined by probit analysis. The differences between those of irradiated and unirradiated mice in each experiment are plotted in Fig. 1. In 16 groups of mice challenged 4 weeks after exposure to 75 r, the LD_{50}'s exceeded those of their controls by an average of 0.129 log. This increase, though not large, is statistically significant at the level $P < 0.001$. The 95% confidence interval is from 0.070 to 0.187. Exposure to 100 r resulted in a smaller (0.059 log) but still significant ($P < .001$) increase in resistance of mice challenged after the same interval, *i.e.*, 4 weeks post-irradiation. From the results on mice challenged after 50 r, it seems improbable that any effect resulted from exposure to that dose.

Leucocyte counts.[§] Total white counts made by customary methods at intervals on mice exposed to 75 r showed a reduction to 6550 (geometric mean) on the 17th day with gradual return to normal (9-10,000) by the 30th day post-irradiation. This finding suggests that when challenged at the 4th week post-irradiation, their circulating phagocytes probably contained a larger proportion of young cells than did those of their unirradiated controls. Differential counts were not made, nor were bone marrows examined for evidence of regeneration of hematopoietic elements.

Discussion. The results show that by the methods employed a small but significant increase in resistance to an experimental bacterial infection was demonstable during the fourth week following exposure to 75 or 100 r X-radiation. This transient effect is regarded as a manifestation of overcompensation in the course of the natural process of recovery and repair, which for a short time may exceed or "overshoot" the normal level.

The increased resistance to infection following a small dose of X-radiation, though not marked nor of long duration, nevertheless suggests a possible explanation for the phenomenon of acquired radio-resistance since endogenous bacterial infection plays an important role in the death of small laboratory animals exposed to mid-lethal doses of ionizing radiation.

Summary. CF-1 female mice, 9-10 weeks old, were exposed to a small dose of whole body X-radiation (50, 75, or 100 r) and together with unirradiated controls were challenged at various intervals by intraperitoneal injection of graded inocula of *Pseudomonas aeruginosa*. Comparison of the LD_{50}'s (30 days) of irradiated and control mice in each challenge showed a small but significant increase in resistance to the experimental infection during the 4th week following irradiation with 75 or 100 r. Exposure to 75 r was more effective than 100 r. The results suggest a possible explanation of "acquired radio-resistance."

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[§] Leucocyte counts were made by Miss Sally Akan.

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Some Biochemical and Physical Changes Occurring in Experimentally-Inflicted Poultry Bruises.* (26886)

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In previous publications on bruised tissue (9) evidence was presented that the characteristic color alterations in bruised tissue represent definite stages in the various physical and metabolic changes which occur during healing. Furthermore, it was shown that when cattle(10) or chickens(12) were bruised upon a second or third occasion, each successive bruise healed more rapidly than the preceding one. Serological and electrophoretical analyses of rabbit serum following bruising have shown that a humoral factor may be responsible for the accelerated healing of secondary bruises(8) and that injection of streptokinase or trypsin enzymes at site of a bruise enhanced the rate of healing in rabbit bruises(13). Zamecnik, Stephenson, and Cope(21) demonstrated the presence of an amino exopeptidase enzyme in normal lymph, serum, muscle, skin, subcutaneous tissue and erythrocytes of dogs and established that the level of this enzyme rises abruptly in the lymph draining a burned or traumatized tissue. The changes that occur in the concentration of a number of compounds of biochemical interest in the plasma and the damaged tissue following onset of an acute inflammatory response(5), trauma(7,14), or wound(18,20) have been investigated.

The present study was initiated to investigate the proteolytic activities and physical changes that occur at specific time intervals following infliction of bruises in poultry. Some of the factors which affect these changes were also examined.

Materials and methods. Bruising and sampling. Chickens 8 to 10 weeks old and weighing approximately 3 lb were kept in constant temperature houses at 7.2, 21 and 30° C for 5-7 days prior to bruising. The area to be bruised and control (non-bruised areas) were clipped and the birds bruised using a standard technique on the breast or the thigh muscles(12). Control and bruised birds were sacrificed at specific intervals following bruising, and both the bruised and control tissues were immediately excised, sliced, minced, and aliquants used for analyses. *Assay of proteolytic activity.* A homogenate of the bruised or control tissue was prepared at 5° C by blending 25 g of the minced tissue with 75 ml of chilled 0.9% NaCl and removing the insoluble residue (blood cells, nuclei and mitochondria) by centrifugation at about 3° C at 6,000 × g for 20 minutes. The supernatant solutions were assayed for proteinases by the method of Kunitz(16) with 0.5% buffered casein as substrate. The proteolytic activity is expressed herein as optical density (O.D.) increment of the incubated solution for 1 hour minus that of the corresponding blank at zero time. Specific activity was defined as Δ O.D. at 280 mμ per 1 hour per

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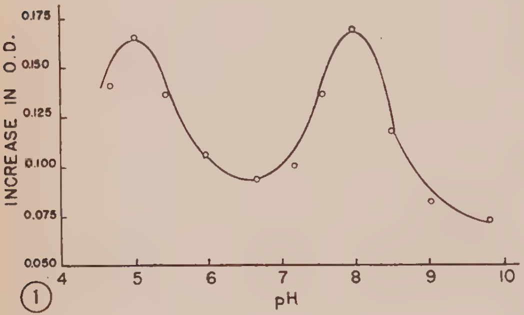
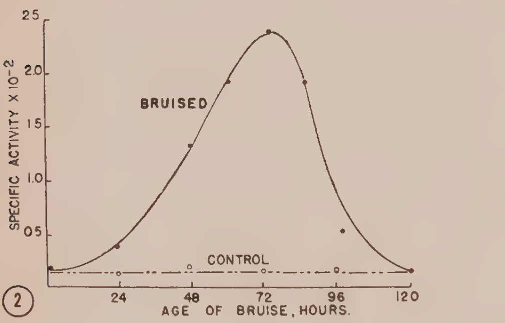
mg protein. The latter was determined by the biuret reaction(6) with crystalline egg albumin as the protein standard. *Pigment extraction.* Aliquants of the minced tissue were subjected to various extraction procedures using saline, buffered to pH 7.4 and containing 1% serum albumin, followed by chloroform, and then alcohol to determine the percent hemoglobin, bilirubin, and biliverdin as described earlier(9,10,11,12). The various extracts were examined spectrophotometrically for extrastromal hemoglobin bilirubin, and biliverdin.





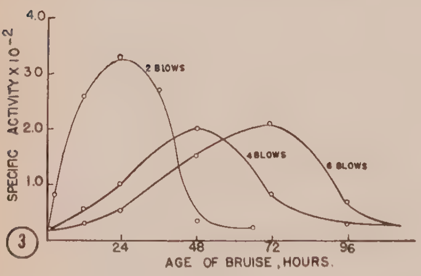


FIG. 1. Hydrolysis of casein by extract of bruised tissue as a function of pH.

FIG. 2. Effect of age of bruise on proteolytic activity present in damaged tissues.

FIG. 3. Effect of severity of bruise on proteolytic activity. Three groups of birds, kept at 7.2°C, were bruised on the breast. The first group received 2 blows, the second 4 blows and the third 6 blows.

Results and discussion. Characterization of proteolytic activity. Fig. 1 shows the effect of pH on hydrolysis of casein by crude extracts of a 2-day-old bruised tissue containing 10 mg protein per ml. The ordinate shows the increase of optical density at 280 mμ for incubation of one hour, and the 2 peaks suggest the presence of at least 2 proteinases in the bruised tissue with pH optima at 5.0 and 8.0, respectively. Heating the bruised tissue homogenate at 80° C for 10 minutes destroyed all the proteinase activity. Action of the plasma cells, the leukocytes, or the autolytic activities of the damaged cells as well as the fluid and serum in the bruised area may be the major sources of these proteolytic activities. Fleisher(4) reported that human leukocytes contain a glycylglycine dipeptidase showing optimal activity at pH 7.7. Koszalka and Miller(15) established that rat skeletal muscle homogenate contained a proteolytic enzyme which readily attacks the endogenous muscle protein at an optimal pH of 8.5 to 9.0. Saline extracts of whole rats and beef lung were found by Dannenberg and Smith(1) to exhibit 2 regions of optimal activity at pH 4.0 and 8.4.

Effect of age of bruise. After the presence of proteolytic activity was established it was decided to examine factors which may affect the proteolysis, optimally active at pH 8.0 in the bruised tissue. Fig. 2 represents typical results of the effect of age and indicates that control and bruised tissue immediately following contusion exhibited the same proteolytic activity. Slight activity was noted 24 hrs after infliction of the bruise, followed by an abrupt increase after 48-72 hours, then a rapid decrease within 72 to 96 hours reaching the control level 120 hours post bruise. Evidence of complete healing, as detected morphologically, was noted from 96 to 120 hours after contusion. The increase in proteolytic activity in the bruised area may be due to the activity of mononuclear phagocytes invading the tissues as well as to the autolysis of the tissue cells. Lymphocytes, monocytes and neutrophils were observed (17) infiltrating livestock bruises one day after bruise infliction and the sarcoplasm exhibited a lytic vacuolization (hydropic degeneration) 2 days post bruise. Wachestein (19) and Fell and Danielli(3) demonstrated evidence of increased enzymatic activity in neutrophils in inflammatory processes. De Duve(2) reported that the lysosomes particles may be involved in intracellular digestion in connection with phenomena of phagocytosis and physiological autolysis or necrosis.

Effect of severity of bruise. The results (Fig. 3) showed that considerable proteolytic activity was detectable within 6-8 hours and reached a maximum after 24 hours, in a superficial type bruise inflicted by using 2 blows, followed by a rapid decrease. Medium (4 blows) and severe bruises (6 blows) exhibited very slight proteolytic activities within 24 hours following bruising. Then a gradual increase to reach a maximum within 48 to 72 hours was observed, with a gradual decrease thereafter. Healing was detected after 2 to 3 days when chickens were bruised superficially, 4 to 5 days when moderately bruised, and 5 to 6 days when severely bruised.

Effect of successive bruising. It has been shown that each of 3 successive bruises in animals(10) or chickens(12) healed more rapidly than did the preceding one. The factor or factors responsible for this accelerated healing were present, at least in the whole blood, and may be passively transferred to a normal animal by transfusion. It was also thought that this successive bruising might affect the proteolytic activity of the injured tissues. Three groups of birds were used in this experiment. Birds of the first group were bruised using 3 blows; some of the birds were sacrificed at 24 and others at 48 hours. The bruised tissues were excised and assayed for proteolytic activity. The second group was treated similarly except each bird was bruised a second time 2 days later using the same applied force. The third group was bruised on 3 occasions 2 days apart. The results are recorded in Table I and indicate that the tissue extract of birds given 2 or 3 separate bruising treatments was more active proteolytically at 24 and 48 hours than the tissue of birds bruised upon only one occasion. The increased activity in chickens previously bruised once or twice may be a result of activation of proteinase synthesis induced by the presence of some factor in bruised tissue, or it may be a direct response to the stimulated phagocytosis following the first bruise. The significant increase in proteolytic activities may account for the accelerated healing on successive bruises as previously established (10,12).

TABLE I. Effect of Successive Bruising on Proteolytic Activities of Tissue.*

No. of birds	Age of bruise	Specific activity × 10^{-2}*		
		1st bruise	2nd bruise	3rd bruise
5	24 hr	.3 ± .05	1.0 ± .20	1.3 ± .30
6	48 "	1.3 ± .20	1.7 ± .30	2.0 ± .30

* Specific activity was defined as Δ O.D. at 280 mμ/1 hr/mg of protein. This value was found to be 0.2 × 10^{-2} for control non-bruised tissue.

Physical changes occurring in poultry bruises. The spectrophotometric absorption curves of the buffered saline and chloroform, and alcohol extracts of control and 2-day-old bruise samples secured from broilers, raised at 7.2, 21 and 30° C, showed that the buffered-saline extract of the bruised area contained hematin and hemoglobin whereas the alcohol extract contained a high concentration of biliverdin. The chloroform extract of bruised tissues secured from birds raised at 30° C showed the presence of a significantly low concentration of bilirubin compared with that obtained from birds raised at 7.2 or 21° C. After the presence of hemoglobin, bilirubin and biliverdin were established in bruised tissue, it was decided to follow quantitatively their changes during healing and also examine the effect of environmental temperatures on the pigments present. The results (Table II) indicate that bruised tissue secured from birds raised at 21 or 30° C accumulated extrastromal hemoglobin which increased in concentration to reach a maximum at 24 hours, followed by a rapid decrease to reach the level of the normal tissue 3-5 days post-bruise. The predominant bile pigment present as a result of hemoglobin degradation(9) was found to be bilirubin in birds raised or kept at 21° C and biliverdin in birds raised or kept at 30° C. These pigments were absent in the damaged areas immediately fol-

TABLE II. Effect of Environmental Temperature on Pigments Present in Poultry Bruises during Healing.

		Concentration in mg/100 g tissue*					
		Environmental temperature					
		21°C			30°C		
Age of bruise in hr	No. of samples	Hemoglobin	Bilirubin	Biliverdin	Hemoglobin	Bilirubin	Biliverdin
½	14	2.0 ± .6	.00	.00	3.2 ± .5	.00	.00
12	12	7.8 ± .7	.7 ± .3	.5 ± .3	8.6 ± .8	.1 ± .05	.9 ± .2
14	13	8.9 ± .7	1.0 ± .4	.5 ± .3	10.2 ± .8	.2 ± .05	1.1 ± .2
18	10	10.6 ± 1.0	1.8 ± .4	.6 ± .2	12.1 ± 1.2	.4 ± .2	2.0 ± .5
24	13	12.8 ± 2.0	2.2 ± .3	.8 ± .3	14.2 ± 2.3	.6 ± .3	3.0 ± .4
48	16	9.2 ± 1.0	2.8 ± .4	1.2 ± .4	6.3 ± 2.0	.8 ± .2	1.6 ± .4
72	12	2.3 ± .5	1.2 ± .4	.6 ± .2	1.0 ± .4	.1 ± .05	.3 ± .1
96	12	.6 ± .2	.5 ± .3	.1 ± .05	.7 ± .2	.00	.00
120	11	.5 ± .2	.00	.00	.6 ± .1	.00	.00

* Normal breast muscle tissue contained .7 ± .2 mg hemoglobin and .00 bilirubin or biliverdin/100 g tissue.

lowing bruise infliction; increased slightly in concentration after 12-14 hours and rapidly after 18-24 hours to reach a maximum after 24-28 hours. They then disappeared from the bruised areas after 96 hours. The accumulation of biliverdin in bruised tissues in birds kept at 30° C may be due to some fundamental difference in the hemoglobin degradation in general or to the impaired synthesis of reducing compounds such as ascorbate or glutathione.

Summary. Proteolytic activities, detected in experimentally-inflicted poultry bruises, were found to be influenced by many factors such as previous bruising, severity, and age of the bruise. Bruised tissues accumulated extrastromal hemoglobin which was degraded primarily to biliverdin in bruises contused on birds kept at 30° C and to bilirubin in birds kept at 21° C or below.

The authors are grateful to Dr. Till Huston, Poultry Dept., for supplying birds raised in the different environmental temperatures.

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Effect of Ascorbate on Healing of Poultry Bruises.* (26887)

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Acceleration of the healing rate of a bruise depends on many factors(4,5) which affect in some way the physical and biochemical reactions of the tissue during the first and second phases of healing. The intense detoxication, protein degradation and renewed biosynthesis, especially that of protein, are among the important factors involved in healing of traumatic injury.

The importance of Vit. C in wound healing is well known. Protein changes, such as collagen synthesis(1,3,13), degradation of hemoglobin(10,11), and formation of bilirubin(4) possibly through reduction of biliverdin, seem to require an abundant supply of ascorbic acid above that usually synthesized by the broiler under normal conditions of growth and handling. This possible need of ascorbate by the injured tissues may be a therapeutic agent to decrease the overall loss and damage due to bruising in poultry. It has been estimated that the loss and damage to the commercial broiler industry due to bruising amounts to 2 million dollars annually in the State of Georgia alone, yet relatively little is known as to how these bruises are inflicted, possible practical and simple means of minimizing the extent of damage, or accelerating the rate of healing of these injured tissues.

Therefore, the studies to be reported here were conducted to investigate the effect of ascorbate on rate of healing and on some biochemical and physical changes occurring in experimentally-inflicted poultry bruises.

Materials and methods. Bruising and sampling. Eight- to 10-week-old birds kept in constant temperature houses were bruised using a standard technic(6). Control and bruised tissues were excised and subjected to various chemical and biochemical analysis as described previously(4,6,7). *Ascorbate.* Total ascorbic acid, defined as the sum of L-ascorbic, dehydroascorbic and 2,3-diketo-l-gulonic acids, was determined by the 2,4-dinitrophenylhydrazine method of Roe and Kuether(16) as modified by Schaffart and Kingsley(17). Ascorbic acid, *i.e.*, the reduced form alone, was determined by the agar diffusion method of Chaiet and Chaiet(2).

Results and discussion. Blood ascorbic acid levels as affected by environmental temperature and bruising. The blood of normal (non-bruised birds 8-10 weeks old) kept or raised at 7.2, 21, and 30°C was assayed for its total and reduced ascorbic acid contents. Another group of birds kept at the same temperatures were bruised and their blood examined 48 hours after contusion. The level of total ascorbic acid in the blood of the control was higher in chickens kept at 7.2 or 21°C than those raised at 30°C (Table I). The reason for this difference is not clear. Ascorbic acid concentration in the blood of birds, 48 hours after bruising, was much lower than that of the non-bruised birds which may indicate a depletion of the ascorbate probably due to its rapid utilization by the injured birds for degradation of hemoglobin present in the bruised area(11), for formation of collagen fibers of the damaged areas(1,3,13,15), or for synthesis and incorporation of "active" hydroxyproline(14). Depletion of ascorbate in the blood of the bruised birds confirms the findings reported by Merezhinskii, Yadvinskaya, and Ivanova (12) on skin burns.

Effect of sodium ascorbate on healing.

TABLE I. Effect of Environmental Temperatures and Bruising on Total Ascorbic Acid Levels in Blood of Chickens.

No. of birds	Temp., °C	Ascorbic acid conc., mg/100 ml Control*	Bruised†
64	7.2	4.80 ± .82	2.60 ± .60
23	21	4.00 ± .80	2.50 ± .60
66	30	3.10 ± .90	1.50 ± .60

* Control non-bruised birds.
† 48 hr post bruise.

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* Approved as Journal Paper No. 151, Coll. Exp. Station, Coll. of Agri., Univ. of Georgia, Athens.

TABLE II. Effect of Injecting Na-Ascorbate on Healing Time and Bilirubin Detected in Poultry Bruises.*

No. of birds	Treatment received (daily)	Healing times (hr)	Bilirubin detection after 12 hr†
12	.2 mg	72 ± 12	slight
12	2 mg	60 ± 12	positive
12	20 mg	72 ± 12	positive
12	No treatment (control)	96 ± 12	negative

* The birds were kept at 7.2°C.

† Conducted on excised tissues.

After the need of ascorbate by the bruised birds above that normally synthesized was demonstrated we examined the effect of injecting sodium ascorbate at site of the bruise on rate of healing of experimentally-induced poultry bruises. Four groups of birds kept at 7.2°C were bruised. Immediately following bruising and daily thereafter, birds of the first group received intramuscular injections of 0.5 ml aqueous solution containing 0.2 mg Na-ascorbate at the center of the damaged areas. The second and third groups were treated similarly except that concentrations of the injected ascorbate were 2 and 20 mg of ascorbate per 0.5 ml aqueous solution, respectively. The fourth group received no treatment. Morphological examination of the bruises before and after killing and detection of tissue bilirubin or biliverdin, a reflection of hemoglobin degradation(5), served as criteria for the effectiveness of the injected ascorbate in promoting or accelerating the rate of healing in poultry bruises.

The results (Table II) indicate that bruises injected daily with 2.0 mg sodium ascorbate healed at a significantly higher rate than observed in birds treated with 0.2 or 20 mg or non-treated bruises. Healing as detected by gross observation occurred within 60 ± 12 hours post bruising whereas the non-treated bruises healed in an average time of 96 ± 12 hours. Bilirubin was chemically detected by the Fouché reagent in the bruised tissue 12 hours after bruising in Na-ascorbate treated birds but not in non-treated bruises. Fast degradation of hemoglobin and accumulation of bilirubin were observed at site of Na-ascorbate injection as measured spectrophotometrically (Table III).

Effect of force applied. A number of birds, kept at 7.2°C, were divided in 3 series and breast bruised as follows: the first sere were bruised using 1 blow; the second received 3 blows whereas the third received 5 blows. Each sere was again subdivided into 2 groups, A and B; Group A received daily intramuscular injection of 2.0 mg sodium ascorbate in 0.5 ml aqueous solution whereas group B served as non-bruised controls. At intervals bruised birds from each sere and group were examined morphologically before and after killing. The results of this experiment indicated that injection of sodium ascorbate reduced the size of the damaged areas after the first injection as evidenced by a clear zone at site of the injected ascorbate. It is believed that ascorbate favorably influenced regeneration of the damaged tissues probably through activating some enzymes, the synthesis of collagen(1,13) in the bruised areas, or accelerating rate of degradation of extrastromal hemoglobin(4) possibly through

TABLE III. Effect of Sodium Ascorbate on the Extrastromal Hemoglobin and Bilirubin Concentration in Poultry Bruises.

Age of bruise, hr	Concentration in mg/100 g tissue			
	Hemoglobin		Bilirubin	
	Non-treated*	Treated†	Non-treated	Treated
½	2.0 ± .6	2.0 ± .6	.00	.00
12	7.8 ± .7	8.0 ± .8	.75 ± .25	.8 ± .3
18	10.6 ± 1.0	7.2 ± .8	1.8 ± .40	2.0 ± .40
24	12.8 ± 2.0	6.6 ± .8	2.15 ± .30	4.0 ± .40
48	9.2 ± .8	4.2 ± .6	2.75 ± .40	2.0 ± .40
72	2.3 ± .5	.6 ± .5	1.20 ± .40	.00 ± .1
96	.6 ± .2	.5 ± .4	.45 ± .3	.00
120	.5 ± .2	.5 ± .4	.00 ± .1	.00

* Non-treated bruised birds.

† Birds receiving daily inj. of 2.0 mg Na-ascorbate.

coupled oxidation with ascorbate(10).

Effect of ascorbate on pigments present in poultry bruises. Two groups of birds, kept at 30°C, were bruised using a standard technic. One group received daily injection of 2.0 mg Na-ascorbate whereas the other group served as control birds. At various time intervals, the birds from both groups were slaughtered, the bruised tissues excised, and concentration of the extrastromal hemoglobin, bilirubin, and biliverdin in the tissues determined. The results showed that bruised birds receiving daily injections of the Na-ascorbate had a lower concentration of extrastromal hemoglobin 18, 24, and 72 hours after bruising than bruised birds receiving no treatment. Sodium ascorbate treated birds accumulated a high concentration of bilirubin 24-48 hours post bruising rather than biliverdin which normally accumulated in control bruises in birds kept at 30°C(7). The accumulation of bilirubin by the Na-ascorbate treated birds may be due to reduction of the free biliverdin at the γ-methene bridge. The latter is formed from hemoglobin which appears to undergo scission at the α-methene bridge as described previously (4).

Effect of Na-ascorbate on proteolytic activities of bruised tissues. Two groups of birds kept at 7.2°C were bruised; the first group was injected daily with 2.0 mg Na-ascorbate in 0.5 ml distilled water whereas the second group served as control bruised birds. At various intervals during healing, birds from each of the groups were sacrificed, the bruised tissues excised and assayed for their proteolytic activities using casein as a substrate(8). The results showed that ascorbate-treated birds had a much higher proteolytic activity at both 24 and 48 hours post bruising followed by a considerable decrease after 72 hours (complete healing) compared with the non-treated bruises. The latter had lower activities after 24 and 48 hours post bruising and much higher activity after 72 hours. The early increase in proteolytic enzymes *in vivo* following the injection of Na-ascorbate may be due to activation of these enzymes which could be another factor accounting for the accelerated healing of Na-ascorbate treated bruises. Activation of proteolytic enzymes in rat skeletal muscles by glutathione and by cysteine (reducing and sulfhydryl agents) was reported recently by Koszalka and Miller(9). Zannoni and La Du (18) established that inactive p-hydroxyphenylpyruvic acid oxidase can be reactivated *in vitro* by addition of ascorbic acid.

Summary. The level of ascorbate in the blood of normal birds was higher in birds kept at 7.2 and 21°C than in those raised at 30°C. Bruising resulted in a depletion of ascorbate in the blood probably due to utilization by the damaged tissues. Bruises injected daily with 2.0 mg Na-ascorbate healed at a much faster rate than bruises of non-treated birds. This treatment resulted in fast degradation of the hemoglobin present, mostly to bilirubin in birds kept at 30°C which normally accumulate biliverdin. Na-ascorbate reduced the size of the bruise and activated proteolytic enzyme in bruised tissue.

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An Effect of Tolbutamide on Nucleotides of Incubated Whole Human Blood.* (26888)

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Tolbutamide is an oral hypoglycemic agent whose mode of action is still in dispute. If this class of arylsulfonamides acts only in the animal containing some islet tissue(1), then these compounds should have no effect *in vitro* on animal tissue since no insulin-producing tissue would be present to be acted upon. Lundbaek *et al.*(2) noted that tolbutamide added to the isolated rat diaphragm would increase glucose uptake, and Renold *et al.* (3) reported that tolbutamide inhibited ketogenesis by rat liver slices and affected glucose oxidation and lipogenesis from glucose by surviving rat adipose tissue. The present work shows that tolbutamide affects the nucleotide pattern of incubated whole human blood and since this pattern is closely related to glycolysis in the red cell, suggests, but does not explain, an influence on carbohydrate metabolism.

Materials and methods. Blood was collected from 2 donors in such a way that the blood samples contained about 2,000 units of heparin and 350 mg of glucose per 100 ml of blood. Aliquots were placed in 2 tissue culture spinner flasks specially water jacketed to maintain 37° and to one flask in each experiment was added 250 mg tolbutamide (Orinase) suspended in 2.5 ml of saline. Samples were withdrawn from all flasks aseptically at several subsequent time periods and trichloracetic acid extracts were made for subsequent nucleotide fractionation(4).

Results. In Fig. 1 and 2 are shown the concentrations of hypoxanthine (Hx), inosine monophosphate (IMP), and adenosine mono-, di-, and tri-phosphates, (AMP, ADP, ATP) in the control and tolbutamide flasks for the 2 subjects. Since ATP is the key energy compound and since on breakdown it follows the pattern(5):

$$ATP \rightarrow ADP \rightarrow AMP \rightarrow IMP \rightarrow\rightarrow Hx$$

it is apparent that in both experiments the presence of tolbutamide was associated with some breakdown in ATP and buildup of degradation products. At 24 hours the changes have started and by 48 hours they are easily seen.

Other measurements such as glucose, pH, and plasma hemoglobin were made in both experiments. The pH in the tolbutamide samples was as much as .1 pH unit below the corresponding control pH at intermediate time intervals but both control and tolbutamide samples came to the same pH by 48 hours. Glucose utilization under the two conditions was not different.

Discussion. Since in the mammalian red cell, glycolysis is the sole source of energy, ATP buildup must be directly coupled with glycolysis. The effect of tolbutamide in reducing blood ATP levels *in vitro* means either that tolbutamide slows ATP synthesis or accelerates its breakdown. The latter might suggest activation of "latent ATPase" while the former could be explained as analogous to "uncoupling" in respiratory phosphorylation. Either process could be compatible with essentially no change in glucose utilization in this *in vitro* system but would

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not necessarily imply no effect on carbohydrate metabolism in the whole animal. In a sense then, the effect of tolbutamide on blood nucleotides indicates more subtle changes in energy metabolism than can be measured by more conventional determinations such as glucose utilization. The effects shown here have been demonstrated in several different experiments and hence are verifiable if not explicable.

Summary. Fresh human blood collected in heparin and glucose was incubated without and with tolbutamide for up to 48 hours. ATP levels in the tolbutamide samples were less than in the control samples although glucose utilization appeared to be the same. It was concluded that the changes in the blood nucleotide pattern were more subtle indicators of effects of tolbutamide on energy metabolism than more conventional measures such as glucose utilization, but the implications of the findings are not entirely clear.

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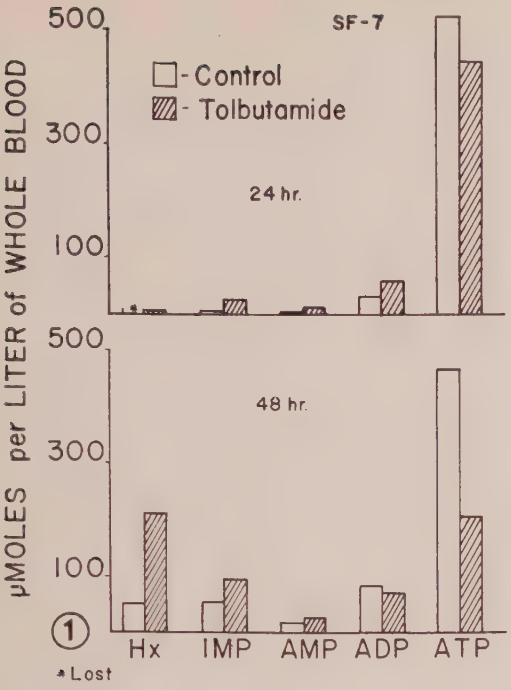

①

FIG. 1. Nucleotide and hypoxanthine levels at 24 and 48 hr in human blood *in vitro* without and with added tolbutamide.

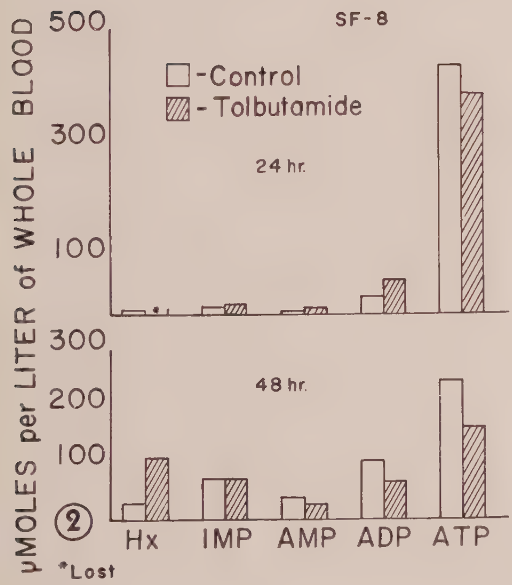

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FIG. 2. Same as Fig. 1 except blood from another subject.

Rate of Utilization of Acetate in the Ruminant.* (26889)

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McClymont(1) identified acetic acid as the major volatile fatty acid in the peripheral blood of the ruminant, and McCarthy *et al.* (2) showed that concentration of blood acetate is directly proportional to concentration of acetate in the rumen. In the present experiments, the rate at which acetate comes from the rumen (and from endogenous sources), the rate at which acetate leaves the body pool, and the rate at which acetate is oxidized to CO_2 in sheep have been investigated using isotope dilution procedures.

In these experiments constant intravenous infusions of radioacetate with fed sheep were carried out since preliminary single-injection experiments did not appear to be interpretable.

Only in the case of the fed animal is there a sizeable acetate pool due to acetate's being absorbed continuously from the rumen. This phenomenon assures an appreciable quantity of acetate in the peripheral blood. Thus, it is under these conditions rather than starvation that we felt it most desirable to carry out the isotope dilution experiments(3). Only very small amounts (1 mg/min.) of acetate were infused in these experiments and served only as a tracer to label the acetate pool.

Experimental. Two yearling grade wethers (weighing 105 and 144 lb), which had been trained to wear a face mask for prolonged periods of time, were used in these experiments. About 3.5 hr before starting infusion, each was fed 1.25 kg of a ration composed of 46.7% chopped alfalfa hay, 47.0% cracked corn, 3.3% soybean meal, 1.0% salt and 2.0% steamed bone meal. The animals had been maintained on this ration for 2 months prior to these experiments. They were given a tranquilizer (tetrahydrozoline, 0.3 mg/lb body wt) 1 hr before the operation, at which time polyethylene catheters (I.D., 0.062 inch, O.D., 0.082 inch) were placed in both jugulars, by a modification of the procedure of Ralston(4), to permit simultaneous infusion and removal of blood samples.

Potassium acetate-C^{14} was infused into the left jugular vein for about 1 hr at a constant rate.‡ The infusion solution was prepared by dissolving 1 mM of potassium acetate, with a total radioactivity of 2.04 mc, in 50 ml of sterile saline. The activity of the resulting solution was 40.8 μc/ml, or 0.02 mM, and 2040 μc/mM, or 4.488×10^9 dpm/mM. The infusion was 2 mc in 60 min. in Experiment 1, and 2 mc in 67 min. in Experiment 2.

Approximately 50 ml of blood were drawn at each withdrawal (20 ml being used for each duplicate assay) through the polyethylene catheter inserted in the right jugular, using a 50-ml syringe fitted with a 16-gauge needle. In Exp. 1, samples of blood were taken just before infusion was begun, at 10-min. intervals during the hour infusion, and at 15-min. intervals thereafter for 45 min. In Exp. 2, samples of blood were taken at 15-min. intervals during the hour infusion, and at 2-min. intervals thereafter for 7 min.

In Exp. 1, the samples were freed of protein by the tungstate procedure of Hawk, Oser and Summerson(5), and the volatile fatty acids in the protein-free filtrate were measured by the procedures of Oppermann *et al.*(6) and Sheppard(7), using a 300 × 12 mm column packed with Celite 535. In Exp. 2, total volatile fatty acids in whole blood were separated by steam distillation in a Markham still (Markham, 8), a method which gave 98% recovery when a standard acetate solution was added to whole blood. Titration of this fraction gave widely varying values, possibly due to the fact that the dis-

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‡ Infusions were performed by use of a continuous automatic infusion-withdrawal pump, Model 600-950, obtained from Harvard Apparatus Co. Inc., Dover, Mass.

tillation was carried out at too low a pH, causing pyruvate to be included in the distillate. Therefore, the distillates were made alkaline and were then concentrated and partitioned on Celite columns as in Exp. 1.

Five-ml eluates from the Celite column were collected quantitatively and placed in scintillation bottles. Five ml of the scintillation solution, prepared by dissolving 6 g of 2,5-diphenyloxazole in 1 liter of redistilled reagent grade toluene, was then added. Radioactivity was measured in a liquid scintillation counter (Packard), and the eluates were then titrated to a bromthymol blue end point with 0.0105 N alcoholic KOH to estimate the acetic acid content.

A face mask, constructed from a metal cup 3 inches in diameter by 2½ inches in depth with a 1 inch diameter T-tube protruding from the end, was used for collection of expired CO_2. The outside of the cup was covered with a piece of rubber innertube, cut to the shape of the face and vulcanized into a tubular shape. The inside of the mask was lined with foam rubber to insure a close fit.

The air to be inhaled by the animal was passed through shell caustic to remove CO_2 and was drawn through the mask at a rate sufficient to maintain a negative pressure to prevent loss of expired air around the closely fitting mask. A one-way flutter valve was situated between caustic and mask.

CO_2 was collected from the expired air by means of 2 glass bubbling towers (150 × 900 mm), each having a sintered-glass disc near the bottom to cause dispersion of the incoming gas through 6 liters of 6.25 N sodium hydroxide. The expired air passed through a flutter valve between the mask and the CO_2 collection towers. Twenty-ml samples of the solution were removed from each tower simultaneously with each collection of blood sample. These samples of the sodium hydroxide solution were analyzed for radioactivity and for CO_2. A 50-μl aliquot was diluted with 6 ml of absolute alcohol and 8 ml of toluene containing 3 g 2,5-diphenyloxazole/liter and counted for 10 min. The amount of CO_2 was determined gravimetrically as barium carbonate.

TABLE I. Acetate Concentration and Radioactivity in Acetate Fraction of Sheep Jugular Blood (Experiment 1).

Time (min.) after starting infusion	Total acetate conc., μc/100 ml blood	Total radioactivity of blood (acetate fraction), dpm/100 ml blood (thous.)	Specific activity of blood acetate, dpm /μM acetate
0	141	0	—
10	148	900	6100
20	103	819	7940
30	136	1183	8680
40	138	1129	8160
50	149	942	6330
60*	149	533	3570
75	125	11	90
90	143	9	60
105	145	7	50

* Infusion stopped.

Results and discussion. Blood acetate. The radioactivity of the various acid fractions measured in Exp. 1 showed that virtually all the radioactivity was in the acetate fraction. The activity of propionate, butyrate and the higher fatty acids was extremely small, usually less than twice background, indicating very little formation from acetate. Although Annison(9) reported finding formate in the blood, it was not found on any of our chromatograms. It was found that a reasonably constant level of radioactivity in the blood acetate had already been reached 10 min. after beginning infusion and that this level was maintained until infusion stopped (Table I). The 50% drop in the 60-min. sample is probably explained by the fact that infusion was stopped some 30 to 60 sec. before the 60-min. blood sample was withdrawn. During this brief period a considerable portion of the radioacetate could have disappeared, as was indicated by the turnover times found.

The data for Exp. 1 indicated that essentially all the radioacetate had disappeared from the jugular blood 15 min. after infusion was stopped. Such radioactivity as was present was presumably coming into the blood as tissue metabolic acetate.

Table II gives similar data for Exp. 2 and shows that the specific activity of radioacetate fell to 1/10th its equilibrium value 4 min. after infusion was stopped. Fig. 1 gives the logarithm of disintegration in min./ml of

TABLE II. Acetate Concentration and Radioactivity in Acetate Fraction of Sheep Jugular Blood (Experiment 2).

Time (min.) after starting infusion	μM/100 ml	dpm/100 ml (thousands)	Specific activity, dpm/μM (thousands)
0	166	—	—
15	123	580	4710
30	155	801	5170
45	178	890	5000
60	108	754	6980
65	225	983	4370
67*	—	—	—
69	222	405	1830
71	466	218	470
74	336	154	460

* Infusion stopped.

blood plotted against time in min. There is a rectilinear decrease during the first 4 min. after stopping infusion, which corresponds to a half-life of about 2.1 min. This corresponds to a turnover time of 3 min. for the body pool $\left(\frac{2.1}{\ln 2}\right)$. This is in good agreement with the value of 3.5 to 4 min. found by Annison and Lindsay(3).

The rate of acetate turnover D, in mM/min., under steady state conditions was calculated as D = i.c.A/a where

D = acetate turnover rate in mM/min.

i = mM radioacetate infused/min.

c = dpm/mM of infused acetate

A = mM acetate/ml of blood

a = dpm in blood acetate/ml of blood.

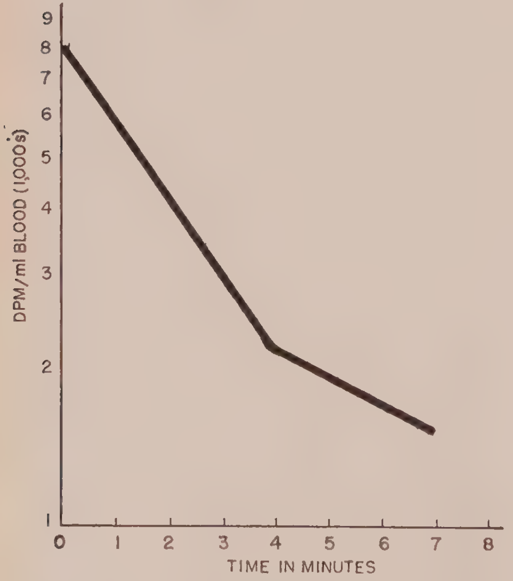

FIG. 1. Disappearance curve for radioacetate in blood.

The acetate concentration in the blood was taken as representative of its concentration in the total "acetate space." Using the average blood values obtained during the equilibrium period, values for rate of acetate turnover in the whole animal were obtained. These are given in Table III.

TABLE III. Rate of Acetate Turnover (Rate of Disappearance of Labeled Acetate from Blood).

Exp.	In mM per min.	In g per hr	In cals per hr	In cals per day*
1	9 7	35	122	2930
2	10.9	39	137	3290

* Calculated on basis of same rate for 24 hr as was found during 3rd-4th hour after feeding.

The "acetate space," V, *i.e.*, size of the body compartment containing acetate in transit under steady state condition can be calculated as follows:

$$V = \frac{D \cdot t\frac{1}{2}}{A \cdot \ln 2}$$

where

V = acetate space in ml

A = mM acetate/ml of blood

D = turnover rate in mM/min.

t½ = half-time of labeled acetate in blood (assumed the same as that in total space).

When calculated, this proves to be 21-22 liters, or about 30% of body weight of the sheep used, in reasonable agreement with figures of approximately 40-45% reported for glucose space in the non-ruminant(10). The acetate space calculated for the weight of the animal minus the rumen would be of the same order.

From the data in Exp. 2 on size of the acetate space and concentration of acetate in the space, as represented by blood concentration, a total pool size of acetate of 33 mM was obtained.

Respiratory CO_2. In both experiments (Table IV) the peak in specific activity of

TABLE IV. Radioactivity of Expired CO_2 from Sheep Given Acetate-1-C^{14} in Jugular Vein.

	Exp. 1		Exp. 2	
Time after starting infusion	% of C^{14} dose in CO_2	Specific activity, dpm/μM	% of C^{14} dose in CO_2	Specific activity, dpm/μM
0	0	0	0	0
10	2.7	2384		
15			6.8	4768
20	5.7	5573		
30	9.3	9234	11.0	8663
40	13.3	13486		
45			13.0	10994
50	16.8	16923		
60	18.4	18412	14.0	13310
75	29.6	22840	22.6	18109
90	38.3	24653	23.4	15631
105		26856	31.6	17628
120	46.8	22967	35.1	18045
135	51.3	22131	37.6	17700
150		19913		
165	57.6	20434		
180	59 2	19545		

respiratory CO_2 occurred almost an hour after infusion was stopped, indicating that equilibrium had never been achieved. Hence, no value for the half life of acetate from time of injection to time of loss of CO_2 in the expired air can be given. However, it was greater than 70 min. (*i.e.*, the approximate time from cessation of infusion to 50% recovery of CO_2) and less than 130 min. in Exp. 1. In Exp. 2 it was also greater than 70 min., but no upper limit can be set in this second experiment due to termination before 50% recovery had been obtained. Thus it appears that acetate, in moving from the blood to its metabolic end products (water and CO_2), passes through intermediary compounds which have much slower turnover rates than does body acetate. The half life of at least 70 min. is longer than that given by Popják *et al.*(11), who reported less than 50 min. in the lactating goat, and that given by Annison and Lindsay(3), who found that most of the CO_2 coming from a single injection of radioacetate was expired by fasting sheep within 1 hr after injection.

The oxidation of acetate occurs *via* the citric acid cycle and requires oxaloacetic acid, which can be synthesized from glucose *via* pyruvic acid and, in the ruminant particularly, from propionic acid *via* succinate. Acetate not metabolized directly to CO_2 and water could serve as precursor of a number of compounds, such as, acetoacetic acid, β-hydroxy butyric acid, higher fatty acids, glycogen, wool fat, cholesterol, and proteins, in the ruminant as it does in other species(12).

Summary and conclusions. Yearling lambs which had been trained to wear a face mask were used in experiments to study the metabolism of acetate. The rate of turnover of acetate in the body, that is, rate of acetate influx from the rumen into the blood, its transfer from the blood into other tissues, its conversion into other compounds, and its subsequent appearance as CO_2 were measured in 2 sheep. Acetate-1-C^{14} disappeared from the body pool with an extremely short turnover time, approximately 3 min., while 50% recovery as expired CO_2 required well over an hour. These results indicate the formation of intermediary compounds which turn over much more slowly than body acetate. The calculated acetate flux during the fourth hour after feeding was 9.7 and 10.9 mM/min/sheep, equivalent to 35 and 39 g of acetic acid/hr.

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Yttrium Localization in the Desert Pocket Mouse *Perognathus penicillatus*.* (26890)

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Since a considerable array of radioisotopes appears in fallout as a result of atomic detonations, there is an increasing need for information concerning localization patterns and factors regulating ion translocation and subsequent deposition. Studies of this nature, however, may involve first, procedures wherein radio-decay leads to formation of other isotopes which may then result in intense localized cellular fields of radiation or lead to stable isotopes which though presenting no radiation problems may behave differently biologically. Thus, the decay of Y^{90} gives rise to radioactive zirconium while Y^{91} leads a stable isotope of zirconium. Differential activity in a biological system may be expected at the molecular level when these 2 isotopes are used, though the final morphological expression may be negligible. Complications in interpretation of localization data may also appear from the fact that little or nothing is known concerning the influence of differential energy spectra or shifts in elemental species on permeability and ion mobility.

Of further interest to biologists are studies concerning rates of turnover of elements in biological systems and pathways in the ecological food chain. Yttrium^{91} has been discovered in fallout from nuclear weapons testing(1) and is believed to follow the distributional patterns of localization of calcium and strontium in vertebrates. In particular, Y^{91} has been found to adsorb on the surfaces of bones(2) and to deposit in small completed Haversian systems. The desert pocket mouse was chosen for this study on yttrium translocation and deposition particularly because this animal appears to be able to thrive in absence of free water and is further thought to resorb water *via* the kidneys, releasing but very slight quantities of urine. Thus, though a large part of injected yttrium is excreted in the urine in some animals within 2 days(3) this opportunity appears to be absent in the desert mouse. It was, therefore, considered of special interest to investigate the distribution pattern of this radioisotope in presence of a reduced mechanism for urinary excretion.

Materials and methods. Adult desert pocket mice, *Perognathus penicillatus* obtained from the desert surrounding Tucson, Ariz. were used. Two males weighing 13.8 g and 15.5 g and 2 females weighing 13.7 g and 15.7 g were used. Yttrium^{91} as YCl_3 in dilute HCl was injected intraperitoneally through the posterior ventrolateral surface of each animal, care being taken to avoid all internal viscera. Amount injected was 18.5 μc in one ml of distilled water per animal. Food consisting of pieces of prickly pear cactus and dry seeds was freely available during the experimental period. After 48 hours the animals were etherized and approximately 0.1 g samples of a spectrum of the major organs and tissues removed. Extraneous radioactivity was removed by washing with running tap water. The pieces were then dried at 80°C, weighed and placed in plastic tubes. Radioactivity was measured by means of a Nuclear Chicago deep well scintillation detector and an ultrascaler. Ten-minute counts were taken in all instances. Appropriate corrections were applied to the data and specific activities were determined in terms of cpm/mg/min. of exposure after initial injection.

Results. The results are summarized in Table I. Although considerable variation was apparent in the comparative accumulation of yttrium in the various organs it remains quite clear that the kidneys retained

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TABLE I. Yttrium Localization in Desert Pocket Mouse, *Perognathus penicillatus*, 48-Hr Accumulation.

cpm/mg/min. × 10^{-4}

	Animal			
Structure	♀ 15.7 g	♀ 13.7 g	♂ 15.5 g	♂ 13.8 g
Brain	.10	.02	4.00	.14
Skin	.05	.59	1.04	17.29
Blood	.35		.38	
Muscle	.39	.11	.22	.52
Diaphragm	1.34	.12	2.56	1.33
Bladder	2.37	.06	1.31	.64
Stomach	3.97	1.35	1.72	2.09
Caecum	3.59	.72	1.86	6.82
Liver	4.20	.89	1.88	7.27
Heart	4.25	.51	.77	5.49
Spleen	4.95	.97	3.45	2.02
Small intest.	6.90	.55	.44	4.73
Tooth	7.95	2.11	3.99	7.39
Gonad	8.68	.00	1.11	2.39
Lung	12.21	.67	.91	3.87
Femur	12.61	.87	3.59	5.34
Kidney	42.80	4.66	10.50	39.96

18.5 μc Y^{91} inj. per animal

the greater amount of activity. The isotope was localized in the tissue of the kidney itself and not in urine since only a piece of this organ was tested from each mouse, and washing procedures were thorough. The teeth and bones were found to have concentrated this isotope also at high levels of concentration. Muscle, blood and brain accumulated but slight amounts of activity except in one female where considerable quantities were apparent in the brain. In one animal, also, amounts of activity in the skin were found to be very high, probably because this particular sample was taken near the site (4,5) of injection.

Discussion. A fundamental difficulty in studies involving ion translocation may exist in the method(6) of administration of the material in question. It is difficult, especially in intraperitoneal injection to be certain that the same injection site is used in each instance. Thus, changes in site might result in considerable variation in subsequent vascular pathways for ion carriage. It has been suggested(7) that the ventral surface of the tail might be an area of choice in isotope injection for outlining the dynamics of ion spread in the bodies of experimental animals. Where this was done in Swiss albino mice, yttrium (Y^{90}) was most actively accumulated initially by the liver and later was translocated. Care was taken in this research to avoid the blood vessels in the tail and to inject relatively large amounts (50 μc) of the radioisotope in small volumes (0.1 ml) into the connective tissue area just under the skin. In other vertebrates (8) Y^{91} has been found to form a colloidal suspension in blood plasma with little apparent accumulation in erythrocytes, and deposition of this ion into visceral and osseous tissue as well as its retention is believed(9) to represent a chemical incorporation of rapidly formed metal-amino acid complexes into various proteins.

Because of the lack of availability of urine in the desert pocket mice it was not possible to check excretion of this cation during the experimental period. In rats, however, it has been shown that about 5% of injected yttrium appears in the urine in 24 hours. Aside from a possible role in supportive structures in vertebrates, a metabolic role for yttrium remains obscure. The concentration of this element in the kidney of the desert pocket mouse probably follows a usual excretory pathway for cations. Subsequent elimination by this means, however, probably does not occur at rates found in non-desert forms.

Summary. Forty-eight hours after intraperitoneal injection of radioactive yttrium (Y^{91}) into the desert pocket mouse *(Perognathus penicillatus)* large quantities of this isotope were discovered in the kidney tissue. Teeth and bones also concentrated this element to a large extent. Very little isotope was found in either blood or muscle.

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Antibody in Hepatitis Patients Against a Newly Isolated Virus. (26891)

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During the past 20 years many investigators have attempted to propagate in the laboratory the etiologic agents of infectious hepatitis and homologous serum hepatitis (1,2).

In the 1940's, several groups of investigators attacked the problems arising from contamination of human plasma with serum hepatitis virus. Their work was concerned in part with studying the effects of heat, irradiation and chemical agents on the virus in contaminated plasma pools. Because of the need for a standard supply of infectious material, the National Institutes of Health (NIH) icterogenic pool was prepared in 1951 from plasma specimens from several patients in the acute phase of clinically typical serum hepatitis(3). Subsequent studies by the group established that parenteral inoculation with this pool material caused hepatitis with jaundice in about 50% of volunteers(3).

This report deals with the preliminary characterization of a cytopathogenic agent recovered on one occasion from primary rabbit kidney cell cultures (RKTC) inoculated with a stored dried aliquot of the 1951 NIH-6* icterogenic plasma pool(3).

Materials. The virus isolate (A-1 agent) was passed serially 15 times in RKTC and subsequently 46 times in primary chick embryo cultures (CETC). Most studies described here were conducted with materials from the 25th to 46th CETC passages. Primary cell cultures were prepared by the conventional trypsinization procedure(4). Chick embryo cultures were prepared in this laboratory; all other cell cultures were obtained from the Tissue Culture Section, Division of Biologics Standards (DBS). Tissue cultures were maintained in either medium 199 or Eagle's Basal Medium (BME) with varying amounts of normal calf serum (NCS) added (0 to 10%). All NCS used in these experiments was inactivated at 56°C for 30 minutes. Animals employed were supplied by the Laboratory Aids Branch, NIH. Embryonated eggs from white Leghorn chickens were obtained from a commercial hatchery. The sources of human sera tested for antibody against the A-1 isolate will be dealt with in a subsequent section. The antiviral sera employed in tests to determine the possible relationship of A-1 virus to known viruses were available in this laboratory, obtained from Dr. Wallace P. Rowe or purchased from Microbiological Associates, Inc.

Methods. A plaque-reduction neutralization test was utilized for all serologic determinations. CETC monolayers in 2 oz. prescription bottles overlayed with our modified 199 plaque medium (Table I) provided the substrate for plaque formation. Sera to be tested were inactivated at 56°C for 30 minutes. Two-fold serum dilutions were prepared in medium 199 containing 10% NCS. The serum dilutions in 0.2 ml amounts were mixed with equal volumes of an appropriately diluted virus suspension which was to contain about 100 plaque forming units (PFU) of virus. After incubation at 36°C for one hour, 0.2 ml from each mixture was inoculated onto the monolayer contained in a single bottle. Following a one-hour adsorption period at 36°C the cell sheets were washed twice with 1.8 ml of medium 199, after which they were overlaid with the agar-plaque medium mixture and incubated at 37°C. Plaque counts were made on the 8th, 10th and 12th days of incubation. Serum neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution causing a 4-fold or greater reduction in plaques, when the control virus mixture contained 60 to 250 PFU. In tests with smaller amounts of virus, *i.e.*, 30 to 60 PFU, an 8-fold reduction was considered significant; tests with less than 30 PFU were deemed unsatisfactory. Serum

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* Aliquots of the NIH infected plasma pool which were stored in the lyophilized state have been coded as NIH-6. The same pool material stored in the frozen state has been referred to in the literature(1) as NIH-8.

TABLE I. Modified 199 Plaque Medium.

	ml
2× concentrated 199 plaque medium (with 1:30,000 neutral red)	72
3% tryptose phosphate broth	9.0
1× Eagle's basal medium (without phenol red)	7.0
2× concentrated skim milk	2.0
	90
3% Noble agar	90
	180

Modified 199 plaque medium and Noble agar are mixed immediately prior to use; 7.5 ml of the mixture is poured into each 2 oz prescription bottle.

dilution values represented the final dilution of serum in the appropriate serum-virus mixture.

Results. Isolation and biologic behavior of agent. The A-1 agent was originally recovered in the following manner: an aliquot of the NIH icterogenic plasma pool was dialyzed against Earle's balanced salt solution for 72 hours, Seitz filtered, heated for 1 hour at 60°C and then inoculated into RKTC. Four days after inoculation cytopathogenic effect (CPE) was observed in the cell culture; transfer of the fluid from this culture demonstrated that it contained a transmissible agent. The uninoculated RKTC control bottles prepared from the same batch of rabbit cells did not develop CPE on incubation. Moreover, 2 blind passages of the control material in RKTC failed to reveal CPE. The A-1 agent produced CPE (Fig. 1) with regularity 4 to 6 days after inoculation even in early passages. The CPE progressed rapidly to complete degeneration of the cell sheet at which time the virus titers of tissue culture supernates were in the range of 10^6 to 10^7 $TCID_{50}/0.2$ ml.

The A-1 virus multiplies in a variety of cell culture hosts. All of the 6 primary and 6 stable cell lines tested to date support its growth (Table II). Titers of 10^6 are common in each of the cell systems listed in the table. Cytonecrosis is seen in each of the cell cultures listed in Table II except in primary rhesus monkey kidney.

Microscopic examination of Giemsa and hematoxylin-eosin stained collodion prepara-

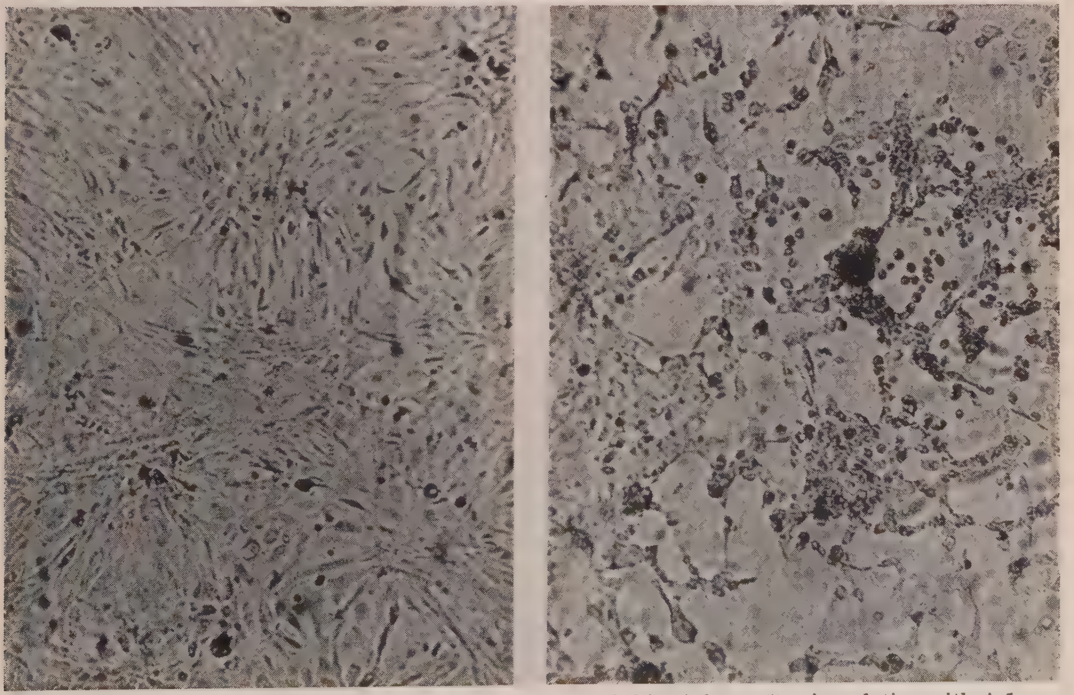

FIG. 1. Primary rabbit kidney—uninoculated control (left); 4 days after inoculation with A-1 agent (right).

TABLE II. Cell Culture Susceptibility to A-1 Agent.

Host cell	Multiplication	CPE
Primary		
Monkey kidney	+	0
Rabbit ”	+	+
Chick embryo	+	+
Human amnion	+	+
Rat embryo	+	+
Cat kidney	+	+
Continuous		
Monkey kidney	+	+
KB	+	+
HeLa	+	+
Human amnion	+	+
Hep-2	+	+
Chang liver	+	+

tions†(5) of infected CETC, RKTC and KB (Eagle) cell cultures revealed no giant cells, inclusions, elementary bodies or other distinctive cytopathological features.

Inoculation of infected cell culture fluids onto blood agar, Sabouraud's agar and in thioglycollate broth gave no evidence of the consistent presence of any bacterial or fungal organisms. Tests for pleuropneumonia-like organisms were negative when a high cell culture passage level of the virus (RK15, CE45) was examined for their presence‡.

Inoculation of the A-1 virus into common laboratory animals and embryonated eggs failed to produce overt disease or gross pathological lesions (Table III). However, blind passage in embryonated eggs established that inapparent infection had occurred. In one instance, after 8 passages in the allantoic sac and in another after 15 in the yolk sac, back titrations of egg materials into CETC revealed amounts of virus comparable to those present in the original tissue culture inoculum (10^6 to 10^7 $TCID_{50}$). Chicks hatched from infected eggs appeared normal. Four serial passages in suckling and weanling mice (IC and IP) showed no evidence of disease and back titrations to cell cultures failed to demonstrate the presence of the virus. Four rhesus monkeys remained healthy during a 6-month observation period following inoculation (Table III). No significant fluctuation was noted in their body temperatures or their white blood cell counts during an 11-week observation period. Tests in these monkeys for viremia and for neutralizing antibody made at weekly intervals for 3 months gave negative results.

Attempts to produce hemagglutination with A-1 virus have been uniformly unsuccessful. Unheated and heat inactivated virus materials obtained from infected RKTC and CETC were mixed with 0.5% suspensions of red blood cells (RBC) from the following animals: man (Groups A, B, and O), monkey (rhesus, cynomologous and vervet), chicken, rabbit, mouse, guinea pig, sheep, hamster, cat and dog. Tubes were observed for 24 hours at 4°, 25° and 36°C. Hemagglutination also failed to occur when RBC from 1-day-old chicks were employed using the method of Havens(7). Infected allantoic and amniotic fluids from the 8th egg passage of the virus likewise produced no hemagglutination.

Physical properties. The A-1 agent retains its infectivity for cell cultures after storage in 2% NCS at –20°C for at least 20 months; lyophilized samples in 10% skim milk kept at room temperature are infectious for at least 16 months. Serum free suspensions of the virus lose their infectivity after 14 days at 4°C, 7 days at 25°C and 1 day at 37°C. Addition of NCS or normal human plasma (NHP) to such virus suspensions preserves infectivity over this temperature range for

TABLE III. Absence of Clinical Disease or Gross Lesions in Laboratory Animals Inoculated with 10^4 to 10^6 $TCID_{50}$ of A-1 Agent.

Animal host	Routes
Weanling mice	IC–IP
Suckling ”	”
” hamster	”
Embryonated chick egg	IC–CAM–YS–All.–Amn.
Newly hatched chick	IM
Guinea pig	IV–IM–IP–SC
Rat	IP–SC
Rooster	IV–IM
Rabbit	IV–IM–SC–Cornea
Monkey	IV–IM–SC–IP–IS–IT

IC = intracerebral; IP = intraperitoneal; CAM = chorio-allantoic membrane; YS = yolk sac; All. = allantoic sac; Amn. = amniotic sac; IV = intravenous; IM = intramuscular; SC = subcutaneous; IS = intraspinal; IT = intrathalamic.

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† Performed by Miss Nancy G. Rogers, DBS.

‡ Performed by Dr. M. F. Barile, DBS, by previously reported technics(6).

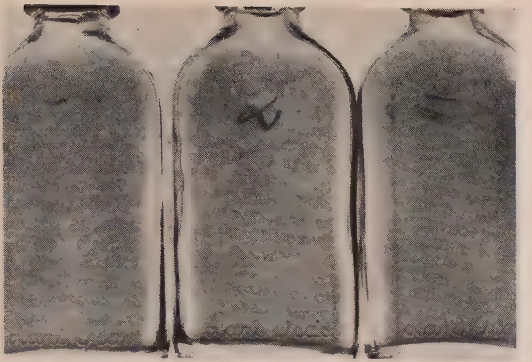

FIG. 2. A-1 agent plaque reduction neutralization test photographed on the 10th day following inoculation and agar overlay of primary chick embryo monolayer tissue cultures.

periods of 3 to 8 weeks. The virus is inactivated in less than 30 minutes at 56°C, even in the presence of undiluted NHP. The A-1 virus is ether and desoxycholate(8) sensitive. It passes through millipore filters of an average size of 300 mμ with no appreciable loss in titer, and also passes through filters of a pore size of 100 mμ but with a marked fall in titer (reduction from 10^6 to 10^1).

CETC fluids containing about 10^6 $TCID_{50}$ of the A-1 virus in serum free medium 199 were tested for stability to photodynamic action.[§] In the presence of toluidine blue (6 μg/ml), polychromatic light from two 100 volt photoflood lamps 7½ inches apart reduced the virus titer below detectable levels in 60 seconds. In contrast to findings with other viruses(9), the addition of either 5% bovine serum albumin or undiluted human plasma so stabilized the virus that, for practical purposes, the titer was unchanged after 10 minutes of irradiation under identical conditions.

Serology. During the course of cell culture passage it was found that the A-1 virus produced discrete plaques in both CETC and RKTC. Since less technical difficulty was encountered with the CETC system, it was chosen for the plaque reduction neutralization test. The results obtained in such a test are illustrated in Fig. 2; the bottle on the left containing the virus control material shows about 60 plaques, that in the middle illustrates a 4-fold reduction of plaques attributable to the neutralizing effect of antiserum, while the bottle on the right illustrates complete neutralization of the challenge dose of virus and no plaques are seen.

The A-1 virus was not neutralized by the various normal and immune sera listed in Table IV.[‖] The 400 human sera mentioned in this table were obtained from a group of people accepted as blood donors.

Human and animal sera contain heat labile inhibitors for the agent. These may be detectable in sera diluted as much as 1 to 80, thus it is essential that all sera used either in media or for serologic testing be inactivated for 30 minutes at 56°C.

TABLE IV. Failure of Various Antisera and Normal Sera to Neutralize the A-1 Agent.

Antiserum	Normal serum	No. tested
Herpes simplex	Rabbit	25
Measles	Rooster	6
Mumps	Rat	8
Poliomyelitis I–II–III	Guinea pig	20
Coxsackie A9	Calf	25
" B1, 2, 3, 4, 5	Monkey	20
ECHO 1–3, 5–19	Human	400
Reovirus 1, 2, 3	Human γ globulin	20
Influenza A, A^1, A^{11}		
" C		
" D		
Parainfluenza 1, 2, 3		
SV - 5		
Lymphocytic choriomeningitis		

Table V contains data on the development of neutralizing antibody against the A-1 virus in 30 persons with experimentally induced homologous serum hepatitis[¶]. The volunteers were from 6 different groups. Each group had been inoculated with material from a different serum hepatitis virus pool during the studies undertaken in man some years ago. One of the 6 represented the

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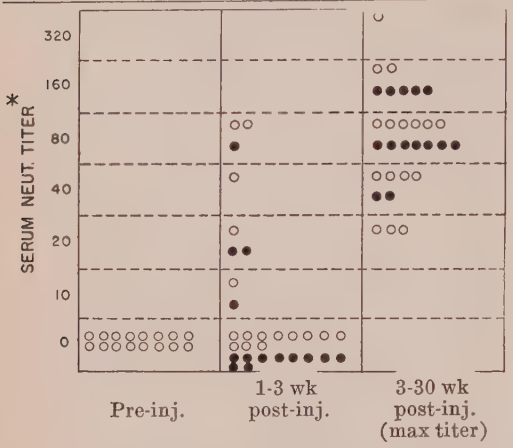
[§] Courtesy Dr. C. W. Hiatt, DBS, by methods previously described(9).

[‖] Great difficulty has been encountered in producing antisera which will neutralize A-1 virus. Hyperimmunization of monkeys, rabbits, guinea pigs, rats and chickens resulted in no detectable neutralizing substances. Low titer sera have been produced in a few guinea pigs inoculated repeatedly with 10^6 $TCID_{50}$ of infectious virus in incomplete Freund's adjuvant. Each of the antisera listed in Table IV was known to contain homologous antibody in high titer.

[¶] Sera kindly supplied by Dr. R. Murray, DBS.

TABLE V. A-1 Agent Antibody in 30 Volunteers Developing Serum Hepatitis.



○ Pre-inj. serum available and tested. ● No pre-inj. serum; first available serum specimen 1 to 3 wk post-inj.

0 neutralization titer = < 10.

* Expressed as reciprocal of serum dilution.

NIH icterogenic plasma pool(3) from which the A-1 agent was subsequently recovered. Four others were icterogenic plasma lots prepared about the same time from individual infected donors(10).** The sixth source of virus was an infected lot of thrombin(11).** Each of these 30 patients developed clinical hepatitis with jaundice. The information in Table V indicates that a 4-fold or greater increase in antibody occurred in all but 2 of these persons. The 2 individuals who failed to show such an increase in antibody did display a 2-fold rise from a titer of 20 to 40 and from 80 to 160, respectively; both were among those from whom the first available serum specimen had been obtained several weeks after inoculation.

Table VI presents serologic results obtained on specimens from 4 different groups of patients whose clinical diagnosis was in each instance infectious hepatitis. These include military personnel who contracted the disease in the United States (IH-I), United Nations personnel who contracted it in Korea (IH-II), a group of American servicemen who became ill after visiting Italy (IH-III) and finally, a group of children who had been inoculated with a strain of infectious hepatitis virus during the course of studies conducted in New York (IH-IV)(12). These groups of sera were graciously supplied by Drs. Ross L. Gauld, J. Anthony Morris, Robert McCollum and Saul Krugman, respectively. The other 17 human sera listed in Table VI were from cases of leptospirosis or leprosy and were included as coded controls. Antibodies against the A-1 virus were found in sera from a small number of individuals but in no instance in which paired sera were tested was a 4-fold or greater rise in titer demonstrated.

Discussion. It should be emphasized that the A-1 virus was recovered on only one occasion following inoculation of cell cultures with the NIH icterogenic plasma pool. It has not been recovered in repeated re-isolation attempts from this same pool, nor has it been recovered from other specimens presumed to contain the virus of homologous serum hepatitis.

Five sera from serum hepatitis cases known to neutralize the A-1 virus at high serum dilutions were absorbed with a suspension made from fresh, frozen, normal human liver. The post-absorption neutralizing antibody titers of these samples were identical with those obtained with the untreated sera. Thus, it appears unlikely that antibody against human liver tissue(13) influences the plaque-

TABLE VI. A-1 Virus Neutralizing Antibodies in Sera of Patients with Infectious Hepatitis and Other Conditions.

Clinical diagnosis	Early sera* No. positive‡ /No. tested	Late sera† No. positive /No. tested	No. with 4-fold rise
IH-I	1/16	2/12	0
IH-II	0/8	0/24	0
IH-III	0/4	9/128	0
IH-IV	0/3	0/3	0
Leptospirosis		0/5	
Leprosy		0/12	

* Early sera = Specimen taken before or up to 2 wk after onset of jaundice.

† Late sera = Specimen taken more than 2 wk after onset of jaundice.

‡ Positive = Neutralization at 1:10 or greater serum dilution.

** The 4 individual plasma sources were from donors designated L.H., V.S., H.H. and C.D., respectively, in Table 4 of reference(10). The thrombin referred to was listed as lot 2 in Table 2 in reference (11).

reduction neutralization test employed.

The plasma aliquot from which the A-1 virus was presumably recovered had been heated for 1 hour at 60°C, yet the tissue culture-adapted isolate is inactivated by similar treatment even when suspended in undiluted normal human plasma. The significance of this observation is undetermined.

Summary. 1. A virus has been recovered in tissue cultures inoculated with the 1951 NIH icterogenic plasma pool. 2. This agent produces cytopathogenic effects in a wide variety of cell cultures, but induces no overt disease in common laboratory animals. It produces inapparent infection in embryonated eggs. 3. Serologic studies have failed to show any relation between the A-1 virus and a number of recognized viruses. 4. Heat inactivated sera from normal human beings and animals did not neutralize the virus. 5. Volunteers infected with serum hepatitis virus from 6 different sources have, with a high degree of consistency, developed antibodies against the A-1 agent. 6. Sera from patients with infectious hepatitis have not neutralized the virus with comparable regularity.

The technical assistance of Alfred Segee is gratefully acknowledged.

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Inactivation of Vacuolating Virus (SV_{40}) by Formaldehyde. (26892)

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Sweet and Hilleman(1) recently reported discovery of a new virus of rhesus and cynomolgus monkey origin. Unlike other simian viruses this agent fails to cause detectable cytopathogenic changes in cell cultures of rhesus and cynomolgus monkey kidneys where it multiplies to relatively high titers. In kidney cell cultures derived from the African Green monkey, *Cercopithecus aethiops*, however, the virus causes cytopathic effects characterized by cytoplasmic vacuolations which coalesce with eventual destruction of the cells. For this reason the virus has been named vacuolating virus and has been designated SV_{40} by Hull.

The present report describes studies on formaldehyde inactivation of SV_{40} and its detection in some lots of poliomyelitis and adenovirus vaccines.

Materials and methods. Tissue cultures. Kidneys obtained from African Green monkeys were trypsinized and grown at 37°C in

Melnick's(2) lactalbumin hydrolysate medium containing 5% heat-inactivated calf serum. The maintenance medium consisted of medium 199(3) pH 7.8 with 2% heat-inactivated chicken serum. The antibiotics used in the media were 100 units of potassium penicillin and 100 μg of streptomycin sulfate per ml.

Virus. Strain 776 of SV_{40} was used in these studies.* Virus pools were prepared in cell cultures of African Green monkey kidneys in serum-free medium 199. The virus pools were stored at −60°C.

Vaccines. Samples of formalinized poliomyelitis and adenovirus vaccines prepared by domestic manufacturers were used during this study.

Virus titrations and serum neutralization tests. Roller tube cultures of monkey kidney cells containing 1.3 ml of maintenance medium were inoculated with 10-fold dilutions of virus. Four tubes were used per dilution and each tube received 0.2 ml of virus. The cultures were observed for cytopathic changes during 14 days of incubation. Neutralization tests were performed by incubating for 2 hours at 37°C a mixture of equal volumes of 200 $TCID_{50}$ of virus per 0.2 ml and heat-inactivated homologous rabbit antiserum.* The virus serum mixture was inoculated in 0.2 ml amounts into monkey kidney culture tubes which were held for 14 days at 37°C.

Formaldehyde treatment of vacuolating virus. Two hundred ml of vacuolating virus suspension were filtered through a Selas 03 filter by negative pressure. The pH was adjusted to 7.0 by addition of 0.1N hydrochloric acid. The pool was divided into 2 portions of 90 ml each and was pre-heated in a water bath to 37°C. To one portion 10 ml of a 1:400 dilution of Formaldehyde Solution USP, was added with constant mixing to produce a final concentration of 98 μg formaldehyde per milliliter as determined by assay. The other portion which received an equivalent volume of balanced salt solution in place of the formaldehyde solution served as a control fluid. The 2 portions were incubated in tightly stoppered flasks in a water bath at 37 ± 0.5°C for a period of 14 days. At various time intervals 5 ml samples were withdrawn and treated immediately with 0.15 ml of 1% solution of sodium bisulfite. The samples were stored at −20°C until they were assayed for virus content.

Detection of SV_{40} in vaccines. Each sample of vaccine was dialyzed at 4°C for 24 hours against 20 volumes of buffered saline pH 7.4 and an additional 24 hours against 20 volumes of medium 199. Two test techniques were employed. In the one, 400 ml of vaccine were concentrated by centrifugation at 30,000 RPM for 3 hours in the Model L Spinco centrifuge with the No. 30 rotor. Gelatin in a final concentration of 0.06% was added before centrifugation to facilitate recovery of the virus(4). The pellets were resuspended in 18 ml of medium 199 and the suspension was inoculated into 3 milk dilution bottles of monkey kidney cell cultures containing 12 ml of maintenance medium. The bottles were incubated at 37°C for 14 days at which time 3 ml from each bottle was subcultured into 6 roller tubes of monkey kidney cells, 0.5 ml per tube containing 1.5 ml of maintenance medium. The tubes were observed for 14 days for any cytopathic changes. In the other technic, 400 ml of vaccine were inoculated into 16 Blake bottle cultures of monkey kidney cells, each receiving 25 ml of vaccine and 50 ml of maintenance medium. After 14 days incubation at 37°C fluids from each bottle were subcultured as described for the concentrated test. In all tests uninoculated cell cultures of the same cell lot were included as controls. The milk dilution and the Blake bottles received 15 ml and 75 ml of maintenance medium respectively. They were observed for 14 days and the fluids were subcultured in the same manner as described for test cultures.

Because of the careful microscopic observation and the high magnification required to detect the characteristic CPE, only the subculture tubes were examined at regular intervals for evidence of the presence of the virus. The primary culture bottles were examined only to assure satisfactory cell maintenance. Final identification of agents encountered in subcultures was done by neutralization tests.

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* Kindly made available by Dr. Hilleman.

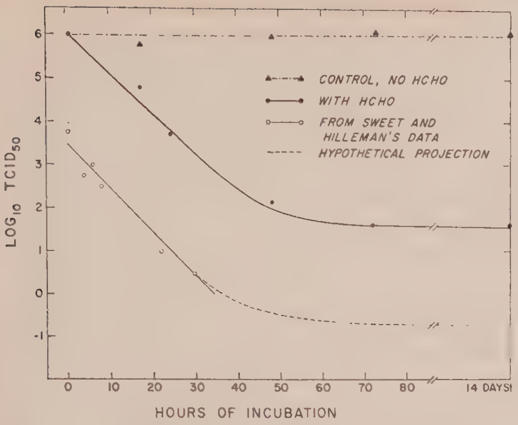

FIG. 1. Inactivation of SV_{40} by 1:4000 formaldehyde solution USP, at 37°C and pH 7.0.

Results. Susceptibility of SV_{40} to formaldehyde. The course of formaldehyde treatment of a serum-free filtrate of SV_{40} suspension is presented in Fig. 1. The slope of the inactivation curve runs approximately parallel to the curve published by Sweet and Hilleman(1) indicating similar rates of inactivation as shown by the solid lines. However, in contrast to the straight line shown by those authors, the results obtained indicate a flattening of the curve with a residual viable fraction remaining throughout the entire 14-day period of observation. A hypothetical projection from the data of Sweet and Hilleman has been included as a dotted line in order to show the probable course of inactivation when lower starting titers of virus are employed. Of further interest is the remarkable thermostability of this agent as evidenced by the full retention of infectivity during heating without formaldehyde at 37° C for a period of 14 days.

Detection of SV_{40} in poliomyelitis and adenovirus vaccines. Randomly selected samples of poliomyelitis and adenovirus vaccines were tested for the presence of viable SV_{40}. The results are summarized in Table I. Four of 8 poliomyelitis vaccines and 3 of 3 adenovirus vaccines were found to produce characteristic cytopathic changes in all subculture tubes. An isolate from each positive vaccine was identified by means of serum neutralization tests. The repeat tests shown were done with different lots of kidney cell cultures and gave similar results. Uninoculated cell controls were included in each test and in addition vaccine D was used as a negative control in the repeat isolations. In no instance was SV_{40} isolated from the negative vaccine control or from the uninoculated cell controls.

To evaluate the sensitivity of the concentrated and unconcentrated test methods all positive vaccines were tested by both methods. No significant difference was observed. With both methods all subcultures derived from positive vaccines showed characteristic cytopathic changes between the 7th-10th day.

Quantitation of SV_{40} in poliomyelitis vaccines. It was of interest to measure the relative amounts of viable SV_{40} in the samples of some vaccines. Ten-fold serial dilutions of 22x concentrated vaccines D, E and F were titrated in tube cultures of monkey kidney cells. At the end of 14 days incubation, fluids from "negative" tubes of each dilution

TABLE I. Detection of SV_{40} in Poliomyelitis and Adenovirus Vaccines.

Vaccines		Test method	Results	Uninoculated cell controls	Identification‡
Polio	Adeno				
A, B, C, D*		Conc.	0/3 †	0/2	
E		Unconc.	16/16	0/3	SV_{40}
E (repeat)		Conc.	3/3	0/2	ND
F		Unconc.	16/16	0/3	SV_{40}
F (repeat)		Conc.	3/3	0/2	ND
G		Conc.	3/3	0/2	SV_{40}
G (repeat)		Unconc.	16/16	0/3	ND
H		Conc.	3/3	0/2	SV_{40}
H (repeat)		Unconc.	16/16	0/3	ND
	I, J, K*	Conc.	3/3	0/2	SV_{40}

* Results shown were obtained with individual vaccine samples.
† No. of positive bottles/Total bottles inoculated.
‡ Isolates identified by neutralization tests.

TABLE II. Titration of Poliomyelitis Vaccine for SV_{40} Content.

Vaccine	Dilutions of concentrated vaccines—$\log_{10}$ 1	2	3	4	Subcultures of pooled negative tubes of each dilution group 1	2	3	4	Estimated virus particles/ml†
D	0/4*	0/4	0/4	0/4	0/4	0/4	0/4	0/4	None
E	1/4	2/4	1/4	0/4	4/4	0/4	4/4	4/4	2000
F	1/4	0/4	0/4	0/4	0/4	4/4	4/4	0/4	200

* No. of tubes showing CPE/Total tubes inoculated.
† Calculated for unconcentrated vaccine.

group were pooled and subcultured without further dilution into 4 tube cultures of monkey kidney cells. Primary titration of formalin-inactivated poliomyelitis vaccine revealed cytopathic changes in only few of the culture tubes (Table II). The results of subcultures from "negative" primary tubes indicated a 10-100-fold higher concentration of SV_{40}. Assuming that only one virus particle was needed to initiate infection of the subculture tubes the number of virus particles in each vaccine was estimated as shown in the last column of Table II.

Discussion. The results obtained in this study indicate that the course of treatment of SV_{40} with 1:4000 formaldehyde was characterized by a biphasic reaction. The major portion of the viral population was inactivated progressively at a slightly slower rate than poliovirus. The second phase of the curve indicated the persistence of a residual fraction which resisted inactivation. The slope of the linear portion of the curve approximately paralleled the one described by Sweet and Hilleman(1). Their failure to detect viable virus after 46 hours of inactivation is undoubtedly due to the fact that the titer of the virus suspension at the start was 2.25 logs lower than the one used in this study. Therefore, the infectivity of the samples taken after 46 hours of inactivation fell below detectable levels when 0.2 ml volumes were used for assay tests. This has been indicated by the hypothetical projection (dotted line Fig. 1) of their curve. Furthermore, Sweet and Hilleman observed their assay tests for a period of only 10 days. In our experience there was a marked delay in the appearance of cytopathic effects caused by formaldehyde-treated SV_{40}. In most instances initial CPE did not appear until the 11th day and the final infectivity titers were reached on the 13th and 14th day. This observation is similar to the findings by Böttiger *et al.* (5) with formaldehyde-treated poliovirus. Hull's(6,7) data on rate of formaldehyde inactivation of the simian viruses known at that time revealed that all of these agents were inactivated more rapidly than poliovirus, with the exception of SV_{12} and SV_{27} which were destroyed at a rate similar to that for poliovirus.

Further studies are required to determine if there is variation in the viral population with regard to susceptibility to formaldehyde or, alternately, if reactivation of "inactivated" particles occurs as described for bacteriophage(8).

In view of the results obtained in the inactivation studies, the presence of viable SV_{40} in killed poliomyelitis and adenovirus vaccines was not entirely unanticipated. The probability that a vaccine lot contains viable SV_{40} in detectable amounts, depends of course on the concentration of this agent before formaldehyde treatment.

In studies designed to estimate the amount of viable SV_{40} present in vaccines, use of a sensitive assay technic was essential. Through subpassage of the "negative" titration tubes at the end of 14 days the sensitivity of the assay was increased up to 100-fold as compared with the primary titration. The estimated quantity of SV_{40} per milliliter of vaccine is in agreement with the results shown in Fig. 1. Since the incidence of SV_{40} infection in kidney cell cultures has been shown to vary significantly with the species of monkeys (1), this factor could influence the presence of detectable SV_{40} in formalinized vaccines. In addition, the degree of contamination of the seed virus inoculum with SV_{40}, the total incubation period of the kidney cell cultures during vaccine production and specific variations in the manufacturing process may also be contributing factors.

Preliminary results(9) of studies on antibody to SV_{40} in normal monkey sera show that rhesus monkeys were infected with SV_{40} as far back as 1956. This suggests that kidney cell cultures and hence Salk poliomyelitis vaccine may have contained the virus in viable form. The finding(1) that a high percentage of persons following injection of 2 doses of poliomyelitis or adenovirus vaccine developed relatively high antibody titers to SV_{40}, prompted Sweet and Hilleman to comment on the apparent high potency of SV_{40} antigen in these vaccine preparations. In retrospect, therefore, one can assume that large groups of the population in this country and abroad must have been injected with varying amounts of SV_{40} during the course of immunization with formalinized poliomyelitis or adenovirus vaccine. Careful clinical studies on selected groups of vaccinees suggest that no harmful effect can be attributed to vacuolating virus within the limits of the observation period. Furthermore, oral† or intranasal(10) administration of SV_{40} in man results in viral multiplication without causing illness. While short term studies of the pathogenicity of SV_{40} in laboratory animals have been reported as negative(1), the long term effect in newborn animals remains to be determined.

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† Melnick, J. L., personal communication.

Summary. Inactivation of SV_{40} with formaldehyde followed a biphasic curve with a levelling off after about 40 hours at 37°C leaving a residual fraction of virus which was resistant to further inactivation for the remainder of the 14-day observation period. Viable SV_{40} was detected in some lots of formalinized poliomyelitis and adenovirus vaccines.

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Plasma and Tissue Electrolytes of Rats Given Excess Sodium Chloride in Food.* (26893)

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Elevated blood pressure and changes in electrolytes have been reported in rats given increased quantities of sodium chloride(1). The present study further characterizes the distribution of sodium, potassium, and water in plasma and tissues of rats ingesting excess sodium chloride.

Methods and materials. Sodium chloride was administered as 2.8, 5.6 and 8.4% of a synthetic diet(2) to 3 respective groups totaling 35 rats. Rats were Sprague-Dawley strain males, each weighing 160 to 235 g at onset of the experiment. Tap water was provided *ad libitum.* Blood pressures were determined by the tail-cuff method(3) under light ether anesthesia and body weights re-

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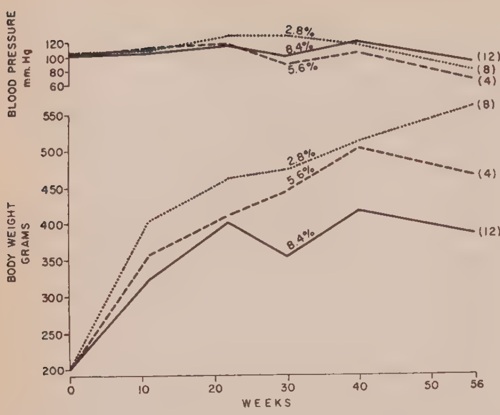

FIG. 1. Changes with time of mean systolic blood pressure (at top) and mean body wt (at bottom) of 3 groups of rats given respectively 2.8, 5.6 and 8.4% NaCl in diet. No. of rats in groups shown in parentheses.

corded every 10 weeks. After 56 weeks on diets rats were stunned and exsanguinated. Gastrocnemius muscle, ventricular heart muscle and aorta (thoracic and abdominal) were removed, immediately dissected free of gross fat and connective tissue and placed in stoppered vessels. Tissues were analyzed by the method of Lowry and Hastings(4), except sodium and potassium were determined by flame photometry. Calculations of extracellular and cellular water phases were based upon extracellular location of chloride.

Carcasses were dried at 105°C for 5-7 days, then ground until they could be sifted through No. 16 mesh. Duplicate samples were subjected to 2 cold petrol ether extractions; differences in dry weight before and after were recorded as neutral fat content. Decanted ether was returned to each vessel containing carcass aliquot, evaporated to dryness and electrolytes extracted with 0.75 N HNO_3 for 24 hours. Before analysis of sodium and potassium by flame photometry, calcium was removed by a modification of Bergstrom's technic(5).

Results. Fig. 1 shows changes in body weights and blood pressures. Failure to gain weight correlated with increasing quantities of sodium chloride in the diet. Compared to rats on 2.8% sodium chloride, fluid intake was increased 2½ times in rats on 8.4% and about 1½ times in rats on 5.6%. Food intake did not differ between groups. No significant elevation of mean blood pressure occurred in any of the 3 groups nor was there any significant elevation of blood pressure of any individual rat. Eight of 10 rats ingesting 2.8%, 4 of 11 ingesting 5.6%, and 12 of 14 ingesting 8.4% sodium chloride in food survived 56 weeks. Low survival rate of animals ingesting 5.6% sodium chloride was attributed to a respiratory infection during the 30th week.

Water and electrolyte values are shown in Table I. With increasing amounts of sodium chloride in the diet, changes in total body potassium did not occur. Total body sodium in rats on 8.4% sodium chloride showed an insignificant increase compared with those on 2.8%. Alterations of potassium and chloride levels of plasma and changes in heart and skeletal muscle electrolytes were not statistically significant. In contrast, aorta showed statistically significant changes in rats ingesting 8.4% sodium chloride compared with those on 2.8%. These consisted of increases in sodium, potassium and chloride, and a decrease in water contents.

Discussion. Increases of total exchangeable sodium in rats on 8.4% sodium chloride and increases in total body sodium and total body potassium in rats on diets high in sodium chloride have been reported(1). In the present study with increased quantities of dietary sodium chloride, total body sodium and potassium were not significantly changed.

Slight increases were reported in sodium and potassium contents of heart and skeletal muscle of rats fed diets containing 5.0% sodium chloride(6). Sodium contents of heart and skeletal muscle increased in rats fed diets containing 10% sodium chloride and either 0.7% or 5.6% potassium chloride(7). No statistically significant changes in heart and skeletal muscle electrolytes were found in the present study.

Increases of sodium, potassium, chloride or water contents of aorta have been noted in rats made hypertensive by a variety of methods, but increased aorta potassium has been most consistent(8). Conversely, decreased potassium content of aorta has been associated with decreased blood pressure incident to reserpine administration to rats previously

TABLE I. Water and Electrolyte Contents of Skeletal Muscle, Heart Muscle, Aorta, Plasma, and Whole Animal.

	Plasma			Whole animal	
	Na	K	Cl	Na	K
2.8% NaCl (8)	146 ± .8	5.0 ±.6	103 ± 1	49 ± .6	47 ± 1
5.6% " (4)	145 ± .7	4.0 ±.2	108 ± 2	45 ± .5	47 ± 1
8.4% " (12)	148 ±1	3.8 ±.2	100 ± .9	52 ±2	48 ± 1

	Na	K	Cl	$(H_2O)t$	$(H_2O)e$	$(H_2O)c$
Skeletal muscle						
2.8% NaCl (8)	42 ± 1	112 ± 3	12 ± .5	790 ± 8	108 ± 4	681 ± 11
5.6% " (4)	41 ± 1	108 ± 2	12 ± .6	770 ± 5	95 ± 6	676 ± 8
8.4% " (12)	38 ± 2	110 ± .8	12 ± .4	786 ± 6	104 ± 3	681 ± 8
Heart muscle						
2.8% NaCl (8)	56 ± 2	85 ± 1	24 ± 1	804 ± 7	213 ±12	590 ± 11
5.6% " (4)	52 ± 1	80 ± 2	24 ± 1	770 ± 6	192 ± 9	578 ± 14
8.4% " (12)	54 ± .4	83 ± .8	24 ± 1	785 ± 6	216 ±11	572 ± 18
Aorta						
2.8% NaCl (8)	115 ±12	42 ± 3	81 ±11	651 ±10		
5.6% " (4)	126 ±14	48 ± 5	80 ±12	632 ±20		
8.4% " (12)	148 ± 7*	52 ± 2*	108 ± 6*	603 ± 9*		

Electrolyte values are expressed as meq/l plasma; meq/kg fat-free wet skeletal muscle, heart muscle, and aorta; and meq/kg fat-free dry whole animal. Water content expressed as g/kg fat-free wet tissue with t = total tissue water, e = extracellular water, and c = cell water.

No. of animals analyzed in parentheses.

± stand. error of mean.

* Statistically significant ($p < .02$) from 2.8% NaCl group.

made hypertensive(9). In our study, increases in sodium, potassium, and chloride contents of aorta were associated with increasing quantities of ingested sodium chloride and not with elevation in blood pressure. This finding of increased sodium, potassium, and chloride in aorta of normotensive rats fed excessive sodium chloride, and in aorta of experimentally hypertensive rats, suggests the presence of a common pathogenetic element other than blood pressure level.

Summary. Rats were fed diets containing excess sodium chloride and examined for changes in water and electrolyte contents. Blood pressure elevation did not occur even after 56 weeks, but body weight varied inversely with the quantity of sodium chloride in the diet. Significant water and electrolyte changes were an increase in sodium, potassium and chloride and a decrease in water content of aorta.

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Influence of Lactogenic Hormone and Estradiol Benzoate upon Placentoma Formation in Intact Rats Measured by Total DNA.* (26894)

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Data on influence of progesterone in stimulating placentoma formation in ovariectomized rat as measured by total DNA have been presented(1). Further, the synergistic influence of estradiol benzoate and thyroxine was observed(2). In normal estrous rat, hormone which would maintain secretory activity of the corpus luteum (progesterone) and produce placentoma should be considered luteotropic. The present study is concerned with the influence of lactogenic hormone (LGH), estradiol benzoate (EB) and combinations of the two upon placentoma formation in intact rat as measured by DNA in order to determine their luteotropic action.

Material and methods. Sprague-Dawley-Rolfsmeyer rats fed Purina Lab Chow were maintained in animal room at 78 ± 1°F until regular estrous cycles were established. When rats showed full estrous smears, 1 mg/day of lactogenic hormone (LGH) was injected for 5 days (pretrauma period). On day 6, one uterine horn of each animal was traumatized as described(1). During post-trauma period, days 6-9, groups received treatment indicated (Table I). On day 10 uteri were separated and processed for DNA (3). Ovine lactogen containing 15 IU/mg was used. Contamination by ACTH, TSH, FSH, LH, and growth hormone was believed to be minimal.

Results. In this study rats which responded by placentoma formation were separated from those not responding. Without treatment during the post-trauma period, 1 of 8 rats responded (Table I). When 1 μg EB was administered, only 1 of 7 rats responded, indicating that EB at 1 μg level has no influence in stimulating placentoma formation.

Lactogenic hormone at each of 2 levels, 1 or 2 mg (15 or 30 IU) stimulated placentoma formation in 5 of 17 rats. In those which responded, DNA/100 g BW of traumatized horns showed significant increases over control horns with 2 mg level exceeding 1 mg level.

When 0.5 μg EB was added to 1 mg LGH, only 1 of 6 rats responded and when EB was increased to 1 μg, 6 of 16 rats showed placentoma formation. Addition of EB to 1 mg LGH increased neither % showing placentoma nor mean DNA/100 g BW of traumatized horns.

In all cases where EB was administered, wet and dry weight of control horns was increased in comparison with those not receiving estrogen. DNA/mg DFFT in traumatized horns was always less than in control horns.

Discussion. In previous experiments, it was shown that administration of 3 mg progesterone (P)/day stimulated maximum placentoma formation as measured by total DNA/100 g BW in traumatized horn of uterus of ovariectomized rats(1). A further increase in DNA/100 g BW in traumatized horn was observed by synergism of P, 6 mg, + EB, 2 μg, + thyroxine, 3 μg/100 g BW (2). The same levels of these 3 hormones have been shown to stimulate increased growth of mammary gland measured by DNA(4). These observations further confirm the suggestion that placentoma formation is primarily influenced by P and that other hormones acted synergically.

In present experiment, normal rats were used to determine what hormones might stimulate P secretion by ovary, and P so pro-

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TABLE I. Effect of Graded Levels of Lactogenic Hormone and Estradiol Benzoate on Placentoma Formation after Traumatization of One Uterine Horn of Intact Rats.

	No. of rats		Wet wt/100 g horn			DFFT/100 g horn		
Treatment*	No reaction	Reaction	Control, mg	Trauma, mg	Increase, %	Control, mg	Trauma, mg	Increase, %
None	7		66 ± 5	83 ± 7	27 ± 8	11.8 ± .8	14 ± 1.2	16 ± 5
		1	48	559	1,072	9.3	75	705
EB: 1 µg	6		112 ± .9	151 ± 14	38 ± 12	18.8 ±1.3	22 ± 1.6	16 ± 9
		1	92	284	209	15.6	42	169
LGH: 1 mg	12		59 ± 3	79 ± 4	37 ± 6	10.9 ± .6	14 ± .1	27 ± 5
		5	50 ± 1	387 ± 70	673 ±148	10.0 ± .4	52 ± 8.4	411 ±71
LGH: 2 mg	12		63 ± 5	90 ± 7	44 ± 8	11.7 ± .9	14 ± 1.1	24 ± 5
		5	52 ± 4	495 ± 66	860 ±126	10.2 ± .9	68 ± 9.0	583 ±94
EB: .5 µg	5		107 ±21	140 ± 22	35 ± 10	17.8 ±3.4	21 ± 2.9	19 ± 5
LGH: 1 mg		1	99	585	500	16.9	83	391
EB: 1 µg	10		94 ± 8	120 ± 9	32 ± 7	15.2 ±1.2	18 ± 1.2	17 ± 5
LGH: 1 mg		6	92 ± 6	376 ± 50	332 ± 88	15.5 ±1.2	53 ± 7.1	263 ±74

			DNA/mg DFFT horn			DNA/100 g horn		
			Control, µg	Trauma, µg	Decrease, %	Control, mg	Trauma, mg	Increase or decrease, %
None	7		42 ± 2.6	34 ± 2.0	17 ± 2.0	.48 ± .026	.47 ±.039	−3 ± .92
		1	56	23	60	.52	1.70	226
EB: 1 µg	6		29 ± 1.1	28 ± 1.5	4 ± 2.5	.54 ± .042	.61 ±.070	12 ± 9.41
		1	28	25	13	.44	1.04	134
LGH: 1 mg	12		45 ± 1.7	36 ± .9	18 ± 2.6	.48 ± .026	.49 ±.024	4 ± 4.54
		5	44 ± 1.9	29 ± 1.3	34 ± 1.0	.44 ± .024	1.48 ±.206	237 ±42.80
LGH: 2 mg	12		45 ± 1.6	32 ± 1.1	28 ± 3.6	.53 ± .045	.46 ±.044	−10 ± .65
		5	45 ± 4.9	26 ± 1.4	41 ± 5.0	.44 ± .044	1.75 ±.201	313 ±58.5
EB: .5 µg	5		31 ± .5	26 ± .6	15 ± 3.5	.55 ± .102	.54 ±.076	2 ± 7.98
LGH: 1 mg		1	47	30	38	.800	2.45	206
EB: 1 µg	10		37 ± .6	33 ± .8	10 ± 3.0	.56 ± .048	.58 ±.044	6 ± .56
LGH: 1 mg		6	33 ± .9	27 ± .6	18 ± 2.7	.51 ± .038	1.42 ±.191	195 ±59.60

LGH = Lactogenic hormone. EB = Estradiol benzoate. DFFT = Dry fat-free tissue.

* Pretrauma period: All rats daily, day 1-5 (6), 1 mg LGH. Posttrauma period: Daily, day 6-9 (7-10).

duced in turn stimulate placentoma formation. Since P showed a graded response measured by DNA, it was thought that level of P secretion might be measured.

It has been suggested that estrogen directly or indirectly *via* pituitary and luteotropic hormone secretion would maintain corpora lutea and P secretion. In present experiment 1 µg EB failed to stimulate placentoma formation.

Evans *et al.*(5,6) were first to present evidence indicating that of various pituitary preparations, their lactogenic preparation containing 30 IU/mg at 1 mg level produced 4 responses in 4 animals and 2 responses in 4 animals at .5 mg level. These observations led to the suggestion that lactogenic and luteotropic hormones were identical.

Present experiment indicates that 1 or 2 mg containing 15 or 30 IU lactogenic hormone stimulated placentoma reaction in about ⅓ of animals (5 of 17), but larger amount increased DNA/100 g to greater extent than smaller dose. The DNA/100 g BW of those responding to higher level of lactogen exceeded DNA of highest level of P administered.

The lactogenic preparation used has been shown by Ferguson and Wallace(7) to contain 17 components by starch gel zone electrophoresis with 3 components making up 70, 22, and 1% of preparation. Pigeon assays indicated 21, 2, and 105 IU/mg lactogen present in these preparations, respectively.

While lactogenic preparation used stimulated marked placentoma formation in about

⅓ of rats, in the light of above observations, authors believe it is premature to identify luteotropic action with lactogen.

It may be pointed out, also, that 1 μg EB used in present experiment stimulated increase in lactogen in 5 days equal to peak level observed on day 16 of pregnancy(8), yet EB had no effect on placentoma formation. If lactogen is luteotropic hormone, it is strange that its concentration is low in early pregnancy when its need for maintenance and secretory activity of corpora is great.

Summary. Sprague-Dawley-Rolfsmeyer rats showing full estrous smears were administered 1 mg/day of ovine lactogen containing 15 IU/mg for 5 days. On day 6, one uterine horn was traumatized and 1 μg EB and 1 and 2 mg lactogen (LGH) were administered on days 6 to 9. On day 10, horns of uteri were separated and processed for DNA. EB had no influence on placentoma formation; whereas, both levels of LGH stimulated placentoma in ⅓ of rats with graded reaction as measured by DNA. EB, .5 μg or 1 μg, with 1 mg LGH improved neither % response nor total DNA in comparison with LGH alone. While lactogenic preparation stimulated placentoma formation, reasons are advanced why it is premature to identify luteotropic action with lactogen.

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Plasma Cholesterol Concentrations in Cockerels and Dogs Treated with Bile Acid Binding Polymer and Cholesterol Synthesis Inhibitors. (26895)

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Plasma cholesterol concentrations in animals and men can be lowered by feeding the bile acid binding polymer, cholestyramine resin (MK-135)[†](1,2), or the hepatic cholesterol synthesis inhibitors, benzmalecene[†] (3,4,5,6), and triparanol (MER-29)[†](7,8). In men the average maximum blood cholesterol lowering achieved with practical doses of these compounds is about 20%. Larger doses are either inconvenient, or produce no greater effect. Bile acid binders and synthesis inhibitors presumably lower cholesterol levels by different mechanisms, and thus combinations of the 2 might produce greater cholesterol lowering than can be attained with either alone. The present work is a study of the action of combinations of cholestyramine resin with benzmalecene and with triparanol in normocholesterolemic cockerels and dogs.

Methods. Cholesterol was determined by the method of Abell *et al.*(9) in plasma prepared by mixing 2.0 ml of whole blood with 0.35 ml of citrate-dextrose (ACD) solution. (Serum cholesterol concentrations may be approximated by multiplying the plasma levels by 1.3.) White Leghorn cockerels, 9-11 weeks old, raised in the laboratory, and fed a mixed natural diet(10), were used for experiments which lasted for 4 days. Treatment was started on Mondays, and blood was drawn for analysis on Fridays. Dogs used were male Beagles, fed a mixture of canned meat and commercial dog meal. Weights and cholesterol concentrations were determined once a week.

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[†] Cholestyramine resin is the generic name for a bile acid binding quaternary ammonium anion exchange resin with styrene-divinylbenzene skeleton. Benzmalecene is the generic name for N-(1-methyl-2,3-di-p-chlorophenylpropyl)-maleamic acid (α-isomer). Both are experimental developments of Merck Sharp & Dohme Research Laboratories, Rahway, N. J., and West Point, Pa. Triparanol is the generic name for 1- [(4-diethylaminoethoxy)phenyl] -1-(p-tolyl)-2-(p-chlorophenyl)ethanol, a product of Wm. S. Merrell Co. Cincinnati, Ohio.

Results. Benzmalecene was given to cockerels by intramuscular injection, either as free acid or as sodium salt, at 100 mg/bird/day in 3 experiments. Cholestyramine resin was fed as 1%, dry weight, of the diet. Average plasma cholesterol concentration, ± standard error of the mean, of 60 controls was 69 ± 1.1 mg %; of 26 birds given benzmalecene, 60 ± 1.6 mg %; of 30 birds fed cholestyramine resin, 53 ± 1.5 mg %; and of 28 birds given benzmalecene plus resin, 49 ± 1.5 mg %. Both substances lowered plasma cholesterol concentrations ($P<.001$) when given alone. Further lowering occurred when they were given in combination. In the same experiments benzmalecene was also given as 0.125% of the diet (about 100 mg/bird/day). Average plasma cholesterol of 30 benzmalecene-fed birds was 71 mg %; and of 30 birds fed benzmalecene plus resin, 56 mg %. Thus, the feeding of benzmalecene did not lower plasma cholesterol, but also did not interfere with the cholesterol-lowering effect of the resin. Increasing the dose of benzmalecene to 1% of the diet, in 3 other experiments, caused decreased food consumption, and loss of weight. Plasma cholesterol levels rose. The increase was comparable to that seen here and elsewhere(11,12) during inanition.

Inasmuch as cholestyramine resin lowered plasma cholesterol of cockerels when fed in the diet, and benzmalecene lowered it only when given by injection, the two substances must exert their effect by different modes of action.

In 4 dogs we have studied the effect on plasma cholesterol of feeding cholestyramine resin (25 g/day, dry weight), sodium benzmalecene (25 mg/kg/day), and triparanol (MER-29) (15 mg/kg/day), alone and in combination. Results with one dog are presented in Fig. 1. Dosing periods generally lasted for 4 to 8 weeks. The order of the different treatments was varied from one dog to another, but this did not influence the results. For all dogs, average cholesterol level during control periods was 101 mg %; with cholestyramine resin, 74 mg %; with benzmalecene, 66 mg %; with benzmalecene plus resin, 41 mg %; with triparanol 42 mg %; and with triparanol plus resin, 15 mg %. All 3 substances lowered plasma cholesterol concentrations ($P<.001$), and further lowering occurred when they were given in combination. On 3 occasions with triparanol plus resin, determined sterol levels were 12, 10, and 9 mg %. Frantz *et al.*(13) and Avigan *et al.*(14) have found desmosterol in the blood of triparanol-treated animals. By our analytical technic pure desmosterol is half as chromogenic as cholesterol. Thus if, during triparanol treatment, all of the plasma sterol were desmosterol, the true concentrations would be twice those reported.

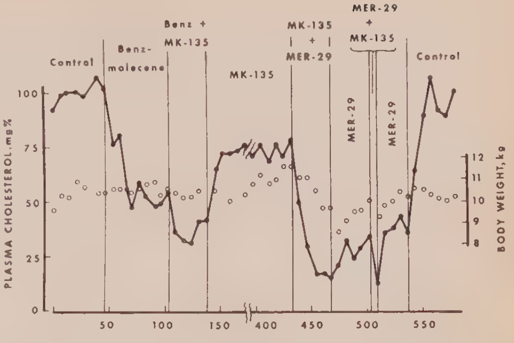

FIG. 1. Plasma cholesterol concentrations (———) and body weights (○) in a dog fed cholestyramine resin (MK-135) (25 g/day), benzmalecene (25 mg/kg/day), and triparanol (MER-29) (15 mg/kg/day).

Toxic signs were detected in dogs with very low blood sterol concentrations. The feeding of resin plus triparanol produced debility, reddened conjunctiva, diarrhea, and anorexia in a dog (Fig. 1) in which plasma cholesterol had already been continuously depressed for 11 months by feeding of resin alone. This dog, and another treated with resin plus triparanol, lost hair at very low blood sterol levels. Their coats improved when treatment was stopped.

Summary. In normocholesterolemic cockerels the bile acid binding polymer, cholestyramine resin (MK-135), lowered plasma cholesterol concentrations when fed in the diet; the hepatic cholesterol synthesis inhibitor, benzmalecene, lowered cholesterol levels when given by injection, but not when fed in the diet. In combination, their effect was additive. In dogs, feeding of cholestyramine resin plus benzmalecene or triparanol (MER-29) had additive cholesterol-lowering effect.

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Phagocytosis of Colloidal Carbon by the Reticuloendothelial System During Hepatocarcinogenesis in Rats. (26896)

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Evidence that the reticuloendothelial system (RES) may be involved in the carcinogenic process has been reviewed by a number of investigators(1,2,3). Several different types of colloids which act directly on the RES have been implicated in the development of tumors(4,5,6). There is also evidence that procedures which stimulate or block the RES will affect the carcinogenic process in animals being fed azo dyes(7,8,9).

It was of interest, therefore, to determine the functional capacity of the RES during the carcinogenic process by measuring the rate of phagocytosis of colloidal carbon. Rates of phagocytosis were determined in rats fed the carcinogens 3′-methyl-4-dimethylaminozobenzene (3′-Me-DAB) or DL-ethionine as well as the basal diets and a weakly carcinogenic azo dye 4′-methyl-4-dimethylaminozobenzene (4′-Me-DAB). A combination of DL-methionine with DL-ethionine was also employed since methionine has been shown to eliminate the liver damage and tumors caused by ethionine(10). Studies with hypophysectomized rats on a 3′-Me-DAB diet were included, since removal of the pituitary gland has been shown to suppress tumor formation in rats fed azo dyes (11). Administration of ACTH or growth hormone will partially restore tumor formation in hypophysectomized rats fed diets containing 3′-Me-DAB(12). Combinations of ACTH and insulin have also been shown to restore tumor formation(13).

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* The authors express their appreciation to Faye Jones for technical assistance.

Methods. Groups of female Holtzman rats were given 0.06% 3′-Me-DAB or 4′-Me-DAB in the semi-synthetic, riboflavin-deficient diet described by Medes *et al.*(14). Basal diets without carcinogen were prepared by the same method and given to a group of control rats. Composition of diets containing 0.6% DL-methionine and/or 0.25% DL-ethionine have been reported by Farber and Ichinose (10). With the exception of one group, all animals on the basal diet were fed *ad libitum.* This group of 12 rats was pair-fed the amount consumed by a similar group receiving 3′-Me-DAB. Since only a slight increase in phagocytosis was noted in the pair-fed animals, the results were included with those obtained under *ad libitum* conditions.

The activity of the RES was determined by slight modifications of the method of Biozzi *et al.*(15). A suspension of carbon (C11/143a)† was injected at the rate of 16 mg/100 g body weight into the femoral vein. The rats were kept under light ether anesthesia during administration of carbon and sampling of the blood. At appropriate intervals blood was collected from the tail vein and a 0.02 ml sample was pipetted into 5 ml hemoglobin diluent (1 g $NaHCO_3$, 50 g KCN, and 200 mg $K_3Fe(CN)_6$ diluted to 1 liter). The concentration of carbon was determined by its absorption at 540 mμ in a Beckman DU spectrophotometer. The blanks were samples of blood obtained prior to injection of carbon. Blood carbon concentration decreased according to an exponential function of time expressed as follows:

$$\log C_1 - \log C_2/T_2 - T_1 = K$$

C_1 and C_2 were the concentrations of carbon in the blood at times T_1 and T_2 respectively. K, which expressed the phagocytic activity of the RES for the injected dose, was called the phagocytic index.

It has been shown that the phagocytic index depends on the weights of the liver and spleen(15). This allows the use of a corrected phagocytic index (α) expressed as:

$$\alpha = \frac{W}{WLS} \sqrt[3]{K}$$

W is body weight and WLS the sum of weights of liver and spleen.

The hypophysectomized rats used in these experiments were Sprague-Dawley females obtained from Hormone Assay Laboratories, Chicago, Ill. One group received daily subcutaneous injections of 0.5 unit of ACTH and 0.0075 unit of insulin,‡ the other group served as a control. Both groups were fed the diet containing 3′-Me-DAB.

Results. Liver weights, phagocytic index (K), and corrected phagocytic index (α) after various times on the azo dyes are shown in Fig. 1. Rats on the basal diet had smaller livers per 100 g body weight than those on any of the azo dyes or groups maintained on Rockland chow. The increase in liver weight after 8 weeks on 3′-Me-DAB coincides with the time when nodules first became visible on the liver.

The phagocytic index of animals fed 3′-Me-DAB increased during the first week and was maintained at high activity through the seventh week. After this time a sharp decline in uptake of colloidal carbon was observed. The phagocytic index of the rats receiving the weak carcinogen 4′-Me-DAB or the basal diet remained low throughout the 15-week feeding period.

Rats receiving the carcinogen 3′-Me-DAB showed an increase in the α value through 7 weeks and then a sharp decline. This decline in the K and α after 8 weeks was not due to inability of the RES to respond to stimulation, since intravenous administration of 5 mg Zymosan§(16) to rats fed 3′-Me-DAB for 10 weeks resulted in marked stimulation of the rate of carbon clearance.

Feeding ethionine also stimulated the rate of clearance of colloidal carbon (Fig. 2).

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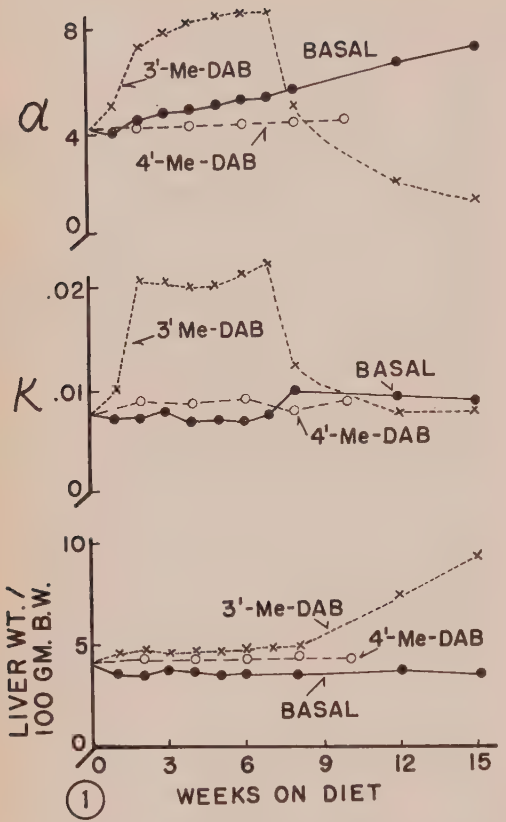
† John Hemschel and Co., Inc., 105 E. 29th St., New York.

‡ ACTH from Orgonon Inc., W. Orange, N. J., Protamine Zinc insulin from Eli Lilly and Co., Indianapolis, Ind. Doses used were suggested in a personal communication from Dr. A. C. Griffin.

§ Lot No. 13335S from K and K Laboratories, Jamaica, N. Y.



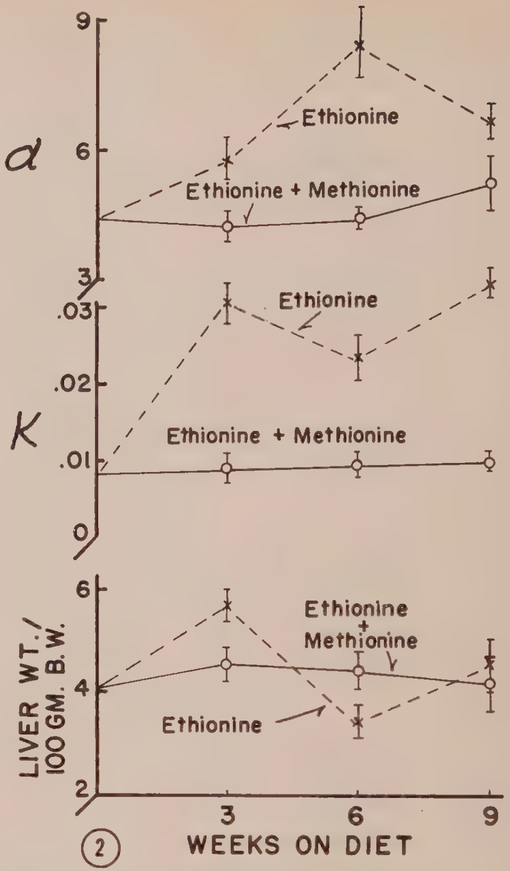


FIG. 1. Liver weights, phagocytic index (K) and corrected phagocytic index (α) of rats fed various azo dyes. Each point is the avg value obtained on from 3 to 6 rats.

FIG. 2. Liver weights, phagocytic index (K) and corrected phagocytic index (α) of rats fed ethionine or ethionine plus methionine. The brackets represent stand. error of the mean value obtained on 4 animals.

When methionine was included in the diet along with ethionine, liver weights and carbon clearance rates remained near normal levels.

Hypophysectomized rats which were fed the diet containing 3′Me-DAB had about the same rate of clearance of colloidal carbon as animals on the basal diet (Table I). When insulin and ACTH were administered to hypophysectomized rats fed 3′-Me-DAB phagocytic activity was again increased.

Discussion. An early increase in the phagocytic index was consistently noted in rats receiving the carcinogens 3′-Me-DAB or ethionine. This increase was not noted in animals fed the basal or weakly carcinogenic diets. It has been shown previously that the effects of ethionine in hepatocarcinogenesis can be partially reversed by methionine(10). The effects of ethionine on phagocytosis of colloidal carbon are also reversed by methionine (Fig. 2). Likewise, it is of interest that the decreased tumor formation after hypophysectomy and the increase after administering insulin and ACTH are paralleled by corresponding changes in the rate of carbon clearance.

The increased phagocytic index between the first and eighth weeks on a diet containing 3′-Me-DAB corresponds quite well with the preneoplastic period. Foci of tumor nodules were consistently noted in all animals after the eighth week of dye feeding and in agreement with others(17,18) tumor incidence at 4 to 6 months was 100%.

Early stimulation of the RES in rats re-

TABLE I. Effect of Hypophysectomy and Administration of ACTH and Insulin to Hypophysectomized Rats on Phagocytic Activity of Animals Fed Diets Containing 3′-Me-DAB.

	Hypophysectomized rats	
Wk on diet	3′-Me-DAB	3′-Me-DAB, insulin and ACTH*
	Phagocytic index—k	
1	.014 (2)†	.006 (2)
2	.007 (2)	.016 (2)
3	.008 (2)	.013 (2)
4	.007 (2)	.014 (2)
5	.005 (1)	.016 (2)
6	.007 (2)	.033 (2)
7	.010 (1)	.031 (1)

* Daily subcut. injections of .5 unit of ACTH and .0075 unit of insulin.

† No. of animals is shown in parentheses.

ceiving carcinogenic diets and elimination of the carcinogenic potency of azo dyes by splenectomy(9) or injections of trypan blue (8), thorotrast or iron oxide(7), suggest an essential role of the RES in hepatocarcinogenesis. Further work will be necessary to establish and clarify the function of the RES in this complex process.

Summary. An early stimulation in the clearance of colloidal carbon by the reticuloendothelial system was noted in rats fed 3′-methyl-4-dimethylaminozobenzene or DL-ethionine. No stimulation was observed in animals receiving the basal diet or 4′-methyl-4-dimethylaminozobenzene. Elimination of the carcinogenic potency of the azo dye by hypophysectomy and of ethionine by methionine abolished the stimulation of phagocytic activity by these carcinogens. Administration of ACTH and insulin restored phagocytic activity in hypophysectomized rats fed diets containing 3′-methyl-4-dimethylaminozobenzene.

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Effect of Age, Temperature and Inoculum Size on Non-Infective Vaccinia Virus Yields in Eggs.* (26897)

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Ratios of 2 biological properties of viruses, such as the ratio of infectivity to hemagglutination units, have been used frequently in studies of the growth characteristics of some viruses. With such technics von Magnus(1) and others(2,3) have demonstrated the appearance of incomplete influenza virus in certain growth situations wherein the proportion of infective virus particles was small compared to the total number of particles as indirectly measured by hemagglutinin titrations. Obviously, a direct total particle count would be preferable but this is a very difficult procedure with influenza viruses. In recent years, methods have been devised which allow rapid and accurate counts to be made of vaccinia virus particles in crude suspensions by electron microscopy so that particle/infectivity ratio experiments with vaccinia virus are feasible(4). Initial application of ratio studies with vaccinia virus was concerned with ratio changes during growth and adaptation of vaccinia virus in eggs, rabbits and guinea pigs(5). Briefly, these experiments demonstrated low ratios of particles to infectious units in serial passages in the chorioallantoic membrane of the embryonated egg which is naturally susceptible to vaccinia virus. However, during adaptive passages in rabbits and guinea pigs high ratios occurred which subsequently dropped as adaptation was accomplished. Thus, during adaptation of vaccinia virus in these animals incomplete virus might be said to have been produced comparable to incomplete influenza virus as described by von Magnus.

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The present study is part of a continuing effort to determine the factors which influence the ratio of vaccinia virus particles to virus infective units and is concerned primarily with attempts to vary this ratio in a naturally susceptible host, the chorioallantoic membrane of the embryonated egg, by allowing vaccinia virus growth to occur under suboptimal conditions. Such conditions included: Incubation of virus infected eggs at below normal temperatures; inoculations of eggs younger and older than the accepted optimal age and passage of virus in high concentrations.

Materials and methods. Virus. Vaccinia virus was obtained from the New York State Board of Health as a calf lymph suspension. It has been subsequently maintained in this laboratory by more than 225 serial passages on the chorioallantoic membrane of 12-day-old embryonated eggs.

Infectivity titrations and virus passage experiments. All growth studies were carried out by inoculation of 0.2 ml virus suspension onto the dropped chorioallantoic membranes of embryonated eggs. Membranes were dropped as described previously(6). At time of harvest eggs were cut open, membranes removed, and extracted to 10% of wet weight. All titrations were made in 12-day-old embryonated eggs as previously described(7) except when specifically indicated. Virus infectious units are expressed in terms of pock-forming units/ml. (PkFU's/ml). All eggs were incubated at the temperature stated ± 1.0°C.

Virus particle counts. This procedure has been described in detail(4,5). In brief, suspensions to be counted were diluted in buffered saline and sonic vibrated at 9 KC for 3 minutes in volumes of 25-50 ml. After sonication the suspension was placed in specially designed centrifugation cells‡ containing an agar block. The cell was placed in a Servall-Sharp‡ counting rotor and centrifuged at 10,000 rpm for 30 min. During this procedure the virus particles were sedimented out of suspension onto the agar block surface. Following this run the block was removed and flooded with collodion which was allowed to dry into a film. The film was stripped off, picked up on an electron microscope grid and examined. Calculation of the number of virus particles was made by the following formula:

$$N = \frac{n\,M^2\,D}{0.97\,(h - t)\,A}$$

N—No. of particles/ml
n —No. of particles per micrograph
M—magnification
D—dilution
h —height of cell
t —thickness of agar
A —area of micrograph

Results. The initial experiment was an attempt to produce relatively large amounts of non-infective vaccinia virus by making serial passages with high virus concentrations since this procedure uniformly results in incomplete influenza virus(1). Table I gives the results and it is apparent that no significant

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‡ Manufactured by Ivan Sorvall, Inc., Norwalk, Conn.

TABLE I. Yields of Infective and Non-Infective Vaccinia Virus after Passage with High and Low Virus Content of Inocula.

	Inoculum					
	1.2 × 10⁷*			1.2 × 10⁴*		
Virus passage	Virus particle count	PkFU's/ml	Particles: PkFU's/ml ratio	Virus particle count	PkFU's/ml	Particles: PkFU's/ml ratio
1	42 × 10⁸	7 × 10⁸	6:1	72 × 10⁸	9 × 10⁸	8:1
2	102 × 10⁸	17 × 10⁸	6:1	119 × 10⁸	17 × 10⁸	7:1
3	161 × 10⁸	23 × 10⁸	7:1	130 × 10⁸	26 × 10⁸	5:1
6	80 × 10⁸	10 × 10⁸	8:1	91 × 10⁸	13 × 10⁸	7:1

* PkFU's/ml = Pock-forming units/ml.

decrease in amount of infective virus resulted. In fact, although a similar procedure with influenza virus results in a marked and progressive infective virus titer decrease in the first 3 passages, vaccinia virus consistently increased for 3 passages. Ratios of total to infective virus were comparable for both concentrated and diluted virus inocula.

Influence of age of eggs on growth of vaccinia virus. Previous experiments in this laboratory have indicated that the age of the chorioallantoic membrane is most important in obtaining maximum titers of vaccinia virus. Table II presents a typical titration in which a stock vaccinia suspension was titered in eggs 8, 12, and 15 days old demonstrating that at both a younger and older age membranes were less responsive to vaccinia virus. This resulted in *apparent* high particle/infectivity ratios of vaccinia virus but actually this was due to non-responsiveness of the membranes involved with consequent decrease in sensitivity to detect infective virus rather than an increase in non-infectious vaccinia.

In another experiment eggs of 8, 12, and 15 days of age were inoculated with vaccinia and the membranes removed and extracted at 48 and 72 hours after inoculation. Extracts were then titered for infective virus in 12-day-old eggs and total particle counts made. Results are given in Table III.

TABLE II. Titration of Stock Vaccinia Virus on Chorioallantoic Membranes of 8-, 12- and 15-Day-Old Eggs Incubated at 37°C.

Age	Virus particle count of stock virus	Infectivity titer of stock virus, PkFU's/ml	Ratio, particles: PkFU's/ml
8	15. × 10⁹	.1 × 10⁹	150:1
12	15. × 10⁹	2.2 × 10⁹	7:1
15	15. × 10⁹	.9 × 10⁹	17:1

Here, in contrast to the previous experiment, it is clear that by deviating from the accepted age of 12 days conditions for vaccinia virus growth are less favorable. The total infective virus yield was about 6-7 times less in both younger and older eggs, but since there was a parallel decrease in total virus particle count the ratios were comparable in all 3 instances, although the single ratio of 10:1 is slightly high.

TABLE III. Growth of Vaccinia Virus in Chorioallantoic Membranes of 8-, 12- and 15-Day-Old Eggs Incubated at 37°C.

Age eggs (days)	Interval inoculation to harvest (hr)*	Virus particle count/ml × 10⁹	Infectious units/ml (PkFU's/ml) × 10⁹	Ratio, particles: PkFU's/ml
8	48	1.3	.19	7:1
	72	8.0	1.3	6:1
12	48	7.3	1.2	6:1
	72	57.0	7.0	8:1
15	48	1.4	.2	7:1
	72	11.2	1.1	10:1

* Inocula = 1.2 × 10⁴ PkFU's/ml vaccinia virus.

Effect of incubation temperature on yield of non-infective vaccinia virus. Since temperature of incubation may influence virus yield a series of growth curves of vaccinia virus were performed in 12-day-old eggs incubated at 26°, 30°, 37° and 41°C. None of the eggs incubated at 41°C survived 48 hours and were discarded from the experiment. Most of the eggs incubated at 26°C survived a maximum of 6 days. Results are presented in Table IV. It is apparent that eggs incubated at 26°C produced virus in an amount

TABLE IV. Yield of Non-Infective Vaccinia Virus in Egg Membranes Incubated at 26°, 30° and 37°C.

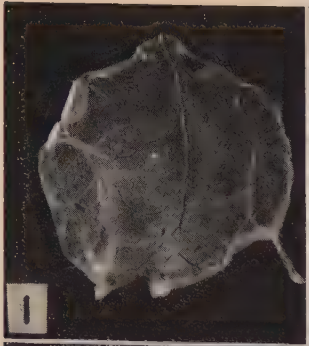
Egg incubation temp. (°C)	Interval inoculation to harvest (hr)	Virus particle count × 10^8	PkFU's/ml × 10^8	Ratio, particles: PkFU's/ml
26	48	.05	.003	17 :1
	72	4.6	.4	11.5:1
	96	5.6	.5	11.2:1
	120	21.0	1.3	16 :1
	144	27	2.7	10 :1
30	48	58	1.8	32 :1
	72	420	30.0	14 :1
37	48	50	10.	5 :1
	72	560	80.	7 :1

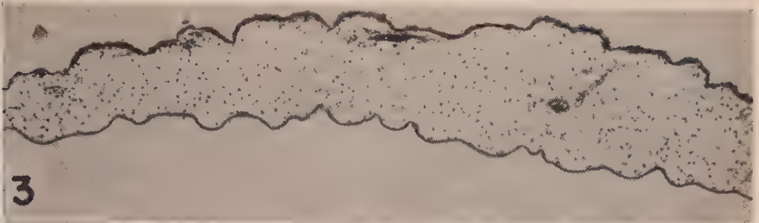
* Inocula for all eggs = 1.2 × 10^4 PkFU's/ml.

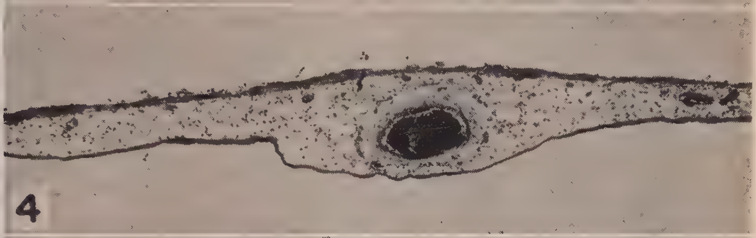
significantly less than at 37°C. With prolonged incubation the virus content increased so that, comparing virus yields at 26° and 37°C, the 3000-fold difference at 48 hours was reduced to a 200-fold difference at 72 hours. The titer of 2.7 × 10^8 at 6 days represents the maximum virus yield obtainable at 26°C; at this temperature all eggs were dead at 7 days. Embryo deaths occurred at 26°C from 96 hours on. Membranes were removed only from eggs containing living embryos. Of further interest was the comparison of gross and microscopic pathology of membranes incubated at 26° and 37°C. Intact membranes which were virus infected and incubated at 26° and 37°C and removed 3 days after inoculation are pictured in Fig. 1 & 2. All eggs received the same inoculum of 1.2 × 10^4 PkFU's/ml vaccinia virus. Careful examination of the 26°C membranes (Fig. 1) failed to reveal any gross lesions of any kind in contrast to the marked lesions found on the 37°C eggs (Fig. 2). Moreover, membranes from the 26°C eggs removed at 4, 5, and 6 days after inoculation were without gross lesions and were identical to the membrane pictured in Fig. 1. Histologic studies of the membranes from virus infected eggs incubated at 26°C (Fig. 4) demonstrated definite cellular infiltration in the choriollantoic membrane but with minimal cell necrosis as compared to marked necrosis found in membranes from eggs incubated at the higher temperature (Fig. 5). It is quite clear from this experiment that the amount of tissue reaction to virus growth may be reduced out of proportion to the reduction in virus multiplication. This is, of course, the situation found with cortisone-treated tissues infected with many bacterial and viral agents. Membranes from eggs incubated at 30°C showed gross and microscopic pathology intermediate between the other two temperatures.

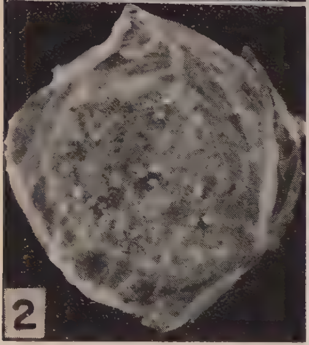
Of primary interest was the increase in proportion of non-infective virus although degree of increase was not as great as had been hoped for and amounted to about a 2-fold increase at 26°C over the average for this type of tissue at 37°C. This increase is significant on the basis of past studies(5). Not included in Table IV are results of titrations of membranes incubated at 26°C but harvested 10, 16, 24, and 36 hours after inoculation, which showed infective vaccinia in titers of 3. × 10^2, 6. × 10^2, 300. × 10^2 and 2000. × 10^2. Ratios could not be calculated on these extracts since the particle concentration was too low for particle counting. These titers did indicate, however, that the virus extracted 10-24 hours and later after inoculation represented virus growth and was not residual virus from the original inoculum.

Discussion. The present experiments demonstrate that it is possible to increase the yield of non-infective vaccinia virus by allowing virus growth to occur under sub-optimal conditions, in this case in the chorioallantoic membranes of eggs incubated at well below normal temperatures. The amount of non-infective vaccinia was measured by the ratio of total particles present as compared to number of infectious units and, in the case of the membranes incubated at the lower temperatures, was found to be increased from an average ratio of 6:1 at 37°C to an average of 13:1 at 26°C. A previously reported average ratio in the chorioallantoic membrane at 48-72 hours at 37°C was 4:1(5) and other unpublished ratios for similar material range from 4:1 to 8:1. Thus, on the basis of the present experiments as well as past experience a ratio of 13:1 represents an increase of non-infective virus of not less than 60% based on the highest (8:1) ratio obtained at 37°C. The standard deviation of the particle count technique is about 10%









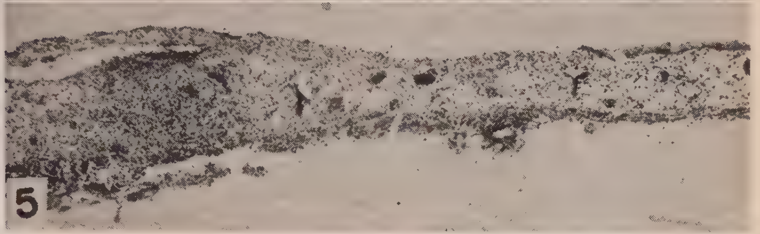


FIG. 1. Membrane inoculated with 1.2 × 10^4 PkFU's/ml of vaccinia virus and removed after 3-day incubation at 26°C. Extracts of this and similar membranes contained approximately 0.4 × 10^8 PkFU's/ml of virus.

FIG. 2. Membrane similar to that in Fig. 1 but incubated at 37°C for 3 days. Virus content about 200-fold greater than those membranes incubated at 26°C.

FIG. 3. Control section of chorioallantoic membrane inoculated with diluent and incubated at 26°C for 3 days. ×60.

FIG. 4. Section of membrane in Fig. 1. Definite increase in cell infiltration with absent or minimal cell necrosis. ×60.

FIG. 5. Section of membrane in Fig. 2. Marked cell infiltration and necrosis in all areas. ×60.

(4) and that of the infectivity titrations 16% (7).

During incubation at 26°C the virus content showed a steady increase over the 6-day period so that none of these determinations represented ratios obtained after peak virus growth was reached. A previous growth curve of vaccinia in chorioallantoic membranes incubated at 37°C and harvested 1 to 6 days after inoculation showed increased ratios beginning at the fourth day after inoculation and associated with both a fall in the infectivity titer and death of the embryos(5). In this latter instance it was presumed that virus inactivation had occurred leading to the increased ratios. Virus inactivation, as opposed to production of inactive or incomplete virus, is difficult to rule out of any experiments of this present type; however, it is apparent that the high ratios obtained at 26°C were found at the earliest interval at which particle counts were possible and showed no tendency to increase as might be expected on a time-temperature inactivation basis.

Few studies of virus growth at subnormal temperatures have been published. Gohd(8) demonstrated growth of influenza virus at 26°C but reduced in amount and delayed in appearance as in the present experiments. Wheeler and Canby(9) stated that herpes simplex virus in tissue culture failed to grow at 26°C and remained in a latent state; later elevation of incubation temperature resulted in a prompt acceleration of virus production. There is no question that vaccinia growth is both delayed and reduced at 26°C. The extent of the pathologic lesions both gross and microscopic was likewise reduced. However, the absolute amount of virus production was considerable. It is surprising that the membrane in Fig. 1 represents a vaccinia virus content of 0.4 × 10^7 PkFU's/ml (extracted) when on the basis of gross pathology the membranes appeared entirely normal. The reduction in pathologic changes at the lower temperatures may be due to a reduced accumulation of virus per cell of the total number of cells infected.

The experiments of vaccinia growth in the 8- and 15-day-old chorioallantoic membrane suggest that a certain rather specific metabolic or physiologic state of these cells is required for maximal virus growth. The chorioallantoic membrane is in this optimal state for only a few days in the total embryonic life span. Some experiments were attempted whereby the 8- and 15-day eggs were incubated at sub-optimal temperatures but this resulted in a virus particle production too low to count. Unfortunately, particle count experiments are not possible (at present) when particle concentration is less than 1 × 10^7 particles/ml.

The mechanism of incomplete virus production is not known but with influenza virus it is produced by serial passage of virus in high concentrations. Since the nucleic acid content of incomplete influenza virus is low it has been theorized that the multiple cell infection strains the cells' ability to produce nucleic acid. No data are available as to whether cell incubation at low temperatures reduces or affects either cell or virus nucleic acid production.

Summary. A relative increase in non-infective or incomplete vaccinia virus occurs during virus growth in the chorioallantoic membrane of 12-day-old eggs incubated at 26°C and 30°C. Vaccinia growth in membranes of eggs 8 and 15 days of age is reduced but no increase in non-infective vaccinia was found. Serial passage of high concentrations of vaccinia virus in 12-day-old eggs likewise failed to increase the proportion of non-infective virus.

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Phosphorylase Activity in Denervated Skeletal Muscle.* (26898)

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The glycogen level of skeletal muscle decreases within 3 days following denervation (1) and returns to normal upon reinnervation(2). However, the changes in muscle phosphorylase activity, following denervation, are not in agreement. Enzyme activity in denervated muscle was reported to decrease slightly after several weeks and then decrease rapidly(3), or to decrease steadily from the day of denervation(4), or to increase up to 20 days after denervation(5). Furthermore, the increase in phosphorylase activity induced in normal skeletal muscle by epinephrine failed to occur in denervated muscle(5). The present study concerns an investigation of skeletal muscle phosphorylase activity at various periods after denervation,

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TABLE I. Phosphorylase Changes in Muscle after Denervation.

		P-lase *a*		P-lase *t*		Ratio
	Days after	Fresh wt[*]	NCPN[†]	Fresh wt	NCPN	*a/t* × 100[‡]
C[§]		125 ± 12	1.87 ± .22	180 ± 14	2.76 ± .25	69 ± 3.3
D[§]	5[‖]	140 ± 13	2.16 ± .12	194 ± 14	2.97 ± .19	73 ± 3.0
C		116 ± 13	1.68 ± .16	178 ± 14	2.58 ± .18	65 ± 2.8
D	10	61 ± 7	.96 ± .09	106 ± 7	1.64 ± .09	56 ± 3.3
C		130 ± 12	2.00 ± .18	190 ± 14	2.89 ± .21	69 ± 2.8
D	15	38 ± 4	.62 ± .04	74 ± 5	1.21 ± .11	51 ± 2.5
C		122 ± 11	1.78 ± .22	188 ± 11	2.72 ± .24	65 ± 3.0
D	20	25 ± 4	.41 ± .07	51 ± 6	.83 ± .11	49 ± 2.9
C		126 ± 6	1.78 ± .12	185 ± 8	2.62 ± .13	67 ± 2.2
D	30	43 ± 3	.70 ± .04	77 ± 5	1.34 ± .13	56 ± 1.3
C		156 ± 12	2.45 ± .20	218 ± 13	3.40 ± .22	71 ± 2.0
D	45	49 ± 4	.86 ± .07	88 ± 8	1.53 ± .15	57 ± 1.7
C		157 ± 9	2.26 ± .14	217 ± 12	3.12 ± .19	72 ± .9
D	60	55 ± 3	.88 ± .05	99 ± 5	1.60 ± .08	56 ± .7

[*] Activity = μg P liberated from glucose-1-PO_4 by 2.5 mg tissue, in 10 min. at 37°C, ± S.E. (7 rats/experiment).

[†] Activity = calculated as mg P liberated per mg of non-collagenous protein nitrogen ± S.E.

[‡] Ratios based on fresh tissue wt.

[§] C = control; D = denervated.

[‖] Results at 5 days not significant, all others significant, $P < .05$.

and the effect of epinephrine on enzyme activity in denervated muscle, particularly after electrical stimulation.

Methods. Adult male Long-Evans rats (250-300 g) were anesthetized with ether and the right rectus femoris muscle was denervated by removal of 2 mm of the femoral nerve. In experiments concerned with enzyme changes at varying periods after denervation the left muscle served as control, and in other experiments bilateral denervation was performed. All subsequent manipulations of the muscles were carried out while the rats were under Nembutal anesthesia. L-Epinephrine-HCl (40 μg/100 g b.w.) was injected intraperitoneally 50 min before autopsy. Tetanic contraction of the rectus femoris muscle was produced by inserting needle electrodes into opposite ends of the exposed thigh muscles and stimulating for 10 or 20 sec from a square wave stimulator set at 60 cycles/min, 1 msec duration, at 20 volts. The muscles were frozen between blocks of dry ice after removal and stored at –20°C until used.

Active phosphorylase *a* and total phosphorylase *t* were assayed as previously reported(6) and the activity ratio (*a/t* × 100) was calculated. The activity is expressed as μg of P liberated from glucose-l-phosphate at 37°C in 10 minutes by 2.5 mg wet weight of tissue or, in some instances, per mg of non-collagenous protein nitrogen (NCPN) determined as described(7).

Results. Activity levels of phosphorylase *a*, *t*, and the *a/t* ratios based on wet weight of muscle, at 5 days following denervation were slightly but insignificantly increased over control values, ($P > .10$). At 10 days phosphorylase *a* and *t* activity decreased significantly in the denervated muscle, but the decrease in activity ratio was of borderline significance. At 15 days both phosphorylase activity and the ratios were significantly lower, and at 20 days these parameters were at their lowest point relative to the controls. From 30-60 days the level of enzyme activity and the activity ratios appeared to increase slightly, but were still much less than control values. No difference in these results was noted when they were based on NCPN (Table I). Attention is called to the increase in phosphorylase *a* and *t* in denervated muscle at 45 and 60 days above the 30-day level together with an increase in the control muscles. However, the activity ratios remained constant from 30-60 days post-denervation.

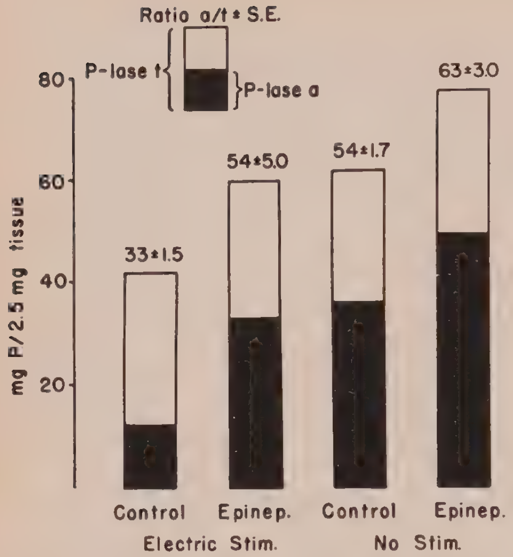

FIG. 1. Phosphorylase activity in denervated (15 days) muscle after electrical and epinephrine stimulation.

In an experiment to determine whether epinephrine increased phosphorylase activity in denervated muscle, rats were bilaterally denervated and after 15 days were divided into 2 groups, one of which received epinephrine injection. Since the effect of epinephrine on muscle phosphorylase is more readily demonstrable when the muscle is made to contract prior to removal for assay(8), in all animals the right leg was electrically stimulated for 20 sec before removal, while the left leg served as a non-stimulated control.

The results (Fig. 1) show that in denervated muscle, which was not electrically stimulated, epinephrine induced a statistically insignificant increase in the activity of both phosphorylase *a* and *t* but a significant increase in the activity ratio. In muscles which were stimulated electrically, epinephrine inhibited the decrease in activity which normally follows stimulation, so that the differences in the activity of phosphorylase *a*, *t* and *a/t* ratio, between epinephrine injected and non-injected animals, were significant.

A study was made of the effect of denervation on rate of recovery of enzyme activity following tetanic contraction, with and without epinephrine treatment. In both intact rats and in animals with muscles bilaterally denervated for 15 days the right leg muscle was stimulated for 10 sec and allowed to recover for varying periods of time before removal. The muscle of the left leg was stimulated for 10 sec and immediately removed. Another group of denervated rats received epinephrine 50 minutes prior to electrical stimulation.

Following electrical stimulation, the activity of phosphorylase *a* decreased in the normal muscle to an extent twice that of the decrease in phosphorylase *t* (Table II). The effect was also noted in a marked decrease in activity ratios. Stimulation of a 15-day denervated muscle produced a significant decrease in both phosphorylase *a* and *t* of approximately the same amount. Tetanic stimulation of the denervated muscle of rats pretreated with epinephrine lowered phosphorylase *a*, *t* and the *a/t* ratio, but only the decrease in the ratios was significant.

In normal muscles the recovery of phosphorylase *a* and the activity ratios occurred within 7.5 min, but the phosphorylase *t* returned in 5 min. In the denervated muscle recovery of phosphorylase *a* and *t* activity was not complete by 20 min, but the activity ratio returned to normal within 5 min. In the presence of epinephrine the return of the activity ratios to their control level in the denervated muscle occurred within 5 min, but since the decrease in phosporylase *a* and *t* themselves was not significant, it was difficult to demonstrate an effect of the hormone on these parameters.

Discussion. The slight increase in phosphorylase *a*, *t*, and ratios of *a/t* which occurred 5 days after denervation of the rat rectus femoris was of questionable significance, although others have reported a significant increase in these parameters as late as 20 days following denervation, using the rat gastrocnemius muscle(5). In another study on this muscle, phosphorylase activity was reported to decrease steadily from the day of denervation(4), while in denervated dog muscle, phosphorylase *b* only was reported and was found to be depressed slightly during the first 7 weeks, after which a marked decrease occurred(3). Such differences may be attributed to differences in the specific

TABLE II. Recovery of Phosphorylase Activity in Denervated Muscle after Electrical Stimulation.

	Initial P-lase activ.†				Recovery of P-lase activ.†		
Exp.*	*a*	*t*	*a*/*t* × 100	Min.	*a*	*t*	*a*/*t* × 100
A. Stim. normal m.	46 ± 2	143 ± 6	32 ± 4.2	2.5	57 ± 2	140 ± 2	41 ± 2.5
				5	90 ± 3	172 ± 6	53 ± 2.4
				7.5	109 ± 11	163 ± 14	65 ± .1
				10	118 ± 9	170 ± 16	69 ± 1.0
(Ref. cont.–Tab. I)				15	122 ± 26	168 ± 34	72 ± 1.0
normal m.	130 ± 13	190 ± 14	69 ± 2.8	20	136 ± 11	190 ± 2	72 ± 4.0
B. Stim. denerv. m.	25 ± 1	57 ± 2	45 ± 1.3	5	31 ± 2	62 ± 2	50 ± 1.5
				10	27 ± 2	52 ± 3	52 ± .7
(Ref. cont.–Tab. I)				15	30 ± 1	54 ± 2	55 ± .6
denerv. m.	38 ± 4	74 ± 5	51 ± 2.5	20	30 ± 1	54 ± 2	56 ± .0
C. Stim. denerv. m. + epineph.	36 ± 2	70 ± 4	51 ± 4.7	2.5	41 ± 1	75 ± 4	55 ± 3.0
				5.0	45 ± 1	72 ± 1	63 ± .9
				7.5	57 ± 2	86 ± 7	65 ± .6
(Ref. cont.–Fig. 1)				10	51 ± 7	75 ± 11	68 ± .3
denerv. m. + epineph.	49 ± 6	77 ± 6	63 ± 3.0	15	46 ± 2	71 ± 6	67 ± 2.0

* Initial determinations (7 rats), recovery determinations (2-3 rats).
† Activity = μg P/2.5 mg tissue ± S.E., as in Table I.

muscle, methods of procuring the muscle, or the enzyme assay.

By the 10th day after denervation phosphorylase *a*, *t*, and the ratios were all lower than in the normal muscles. This condition persisted for 60 days. The decrease in enzyme activity observed agrees with some previous results(3,4) but not with others(5). Phosphorylase assays using tissue homogenates are highly variable at best and interpretations must take into account activity ratios as well as absolute values of phosphorylase *a* and *t*. Muscle atrophy produced by vit. E deficiency(9) and muscular dystrophy from other causes(10) are associated with decreased phosphorylase *a* and *t*, but under these conditions the ratios were similar to those in normal muscle. The decrease in enzyme activity was not necessarily associated with increases in amount of connective tissue relative to amount of muscle protein because the results calculated on the basis of NCPN for denervated muscle were similar.

In normal muscle epinephrine was reported to increase phosphorylase *a* and the activity ratios(11) and to inhibit the expected decrease in enzyme activity which ordinarily follows tetanic stimulation(8). In denervated muscle epinephrine increased only the activity ratios significantly, but prevented the expected decrease in phosporylase *a*, *t*, and the ratios which follow tetanic stimulation. Previous failure to demonstrate the effect of epinephrine on phosphorylase in denervated muscle may have been due to differences in procedures(5). Epinephrine induces glycogenolysis in denervated muscle(1) and it would seem likely that the phosphorylase activity would also increase.

In the experiments on recovery rates of enzyme activity from depressed levels 10 sec electrical stimulation was selected rather than 20 sec because the latter frequently overwhelmed the effect of epinephrine in preventing the decrease in phosphorylase activity. In normal muscles the return of enzyme activity occurred within 7.5 min. In the denervated muscle the return of activity ratios to their characteristic initial levels appeared slightly faster than in intact muscle, but phosphorylase *a* and *t* had not returned within a 20-min. period indicating some impairment in this instance. Tetanic contraction is reported to decrease only phosphorylase *a* by converting it into phosphorylase *b* which can be calculated by difference when phosphorylase *t* is determined(11,12). Although the decrease in phosphorylase *a* accounted in large part for the low ratios, the decrease in phosphorylase *t* cannot be either ignored or explained.

Although others have shown that epinephrine increases rate of recovery of phosphorylase activity ratios in normal rat muscles(11,12), this was not observed in denervated muscle under these conditions. Perhaps if the denervated muscle had been stimulated to exhaustion, the effect of epinephrine on increasing the rate of recovery would have appeared as it did in normal muscle(11,12).

Summary. Following denervation of the rat rectus femoris muscle, phosphorylase *a* and *t* activity and the *a/t* ratio were maintained for 5 days, decreased by 10 days and remained low for 60 days. In muscles denervated for 15 days epinephrine increased the activity of phosphorylase *a* and *t* and the *a/t* ratio, and markedly inhibited the expected decrease in enzyme activity which follows tetanic contraction. Following electrical stimulation, the recovery of activity ratios in both intact and denervated muscles occurred at the same time. In denervated muscle the return of phosphorylase *a* and *t* was delayed as compared to that in normal muscle. Epinephrine showed no effect on the recovery of enzyme activity in the denervated muscle. Changes in muscle phosphorylase activity after denervation atrophy differ from those incurred from other causes.

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Influence of Adrenal Glucocorticoids on Distribution of Calcium and Phosphorus between Bone and its Surrounding Fluids. * (26899)

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Hypercalcemia has been reported in Addisonian crisis(1-3), following adrenalectomy in patients with Cushing's disease(4), and in adrenalectomized animals(5-8). In many of these instances the elevated serum calcium returned to normal levels following cortisone administration. Hypercalcemia accompanying sarcoidosis and hypervitaminosis D, and idiopathic hypercalcemia in children may be similarly reduced with cortisone(9-11).

Elevated serum phosphorus values have been observed in adrenalectomized rats(12) and monkeys(13), and in patients with Addison's disease(2). On the other hand, ACTH has been found to decrease serum phosphorus in the rat(14), and marked hypophosphatemia has been seen in patients following cortisone treatment(15).

Either a lack of such effects on serum Ca and P concentrations or conflicting results have been reported by other workers(11,14,16). While these phenomena indicate effects

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of adrenal hormones on serum Ca and P levels, the mechanisms underlying these effects are unknown.

In recent studies in dogs(8) hypercalcemia following adrenalectomy was reversed to normocalcemia with cortisone. A balance study during the hypercalcemic phase revealed no change in the consistently negative Ca and P balance, suggesting that the effects on serum calcium were not mediated through changes in balance. It was therefore suggested that cortisone, in addition to its well-known effects on calcium balance, may also influence the equilibrium distribution of Ca between bone and extracellular fluid in a direction opposite to that of parathyroid hormone and vit D. A study of the possible influence of adrenalectomy and cortisone treatment on this equilibrium using mouse bone *in vitro* is here reported.

Methods. The experimental animals used were 7-8-week-old, male Swiss white mice weighing 40-45 g each. Adrenalectomy was performed one week before sacrifice. 2.5 mg cortisone acetate Merck was injected subcutaneously as described in Table II. On the day after the last dose was given,[§] animals were killed by cervical fracture and the calvaria removed and prepared as in other experiments(18). Two calvaria, pooled to give about 80 mg wet weight of bone in each flask, were incubated for 6 hours in a Dubnoff metabolic incubator at 37.8°C in 2 ml of modified Krebs-Ringer media[||] buffered to pH 7.4 with HCO_3^- and 5% CO_2. Penicillin at a final concentration of 5 U per ml and streptomycin 0.01 mg/ml were added. Glucose was used as a substrate at a concentration of 2 mg/ml. Incubations were carried out in an atmosphere of 95% oxygen with 5% CO_2. In such a system at the end of 6 hours incubation Ca and P measured in the medium can be considered to be in steady-state equilibrium with the bone mineral and thus simulate the situation *in vivo* discussed elsewhere(18). For each experiment normal and experimental bone samples were incubated at the same time. All analyses were done by methods currently in use in this laboratory (18).

TABLE I. Effects of Adrenalectomy.

		Ca	P	Ca × P
		mMol/l		
Controls	1	.64	.94	.60
	2	.68	.95	.65
	3	.66	.92	.61
	4	.65	.91	.59
	5	.65	.90	.59
	Mean	.66	.92	.61
Adrenalectomized	6	.58	.93	.54
	7	.65	.89	.58
	8	.62	.89	.55
	9	.65	.93	.60
	10	.65	.93	.60
	11	.71	.97	.69
	Mean	.64	.92	.59

Results. Neither adrenalectomy nor treatment with cortisone (or hydrocortisone) in the dosage used appeared to affect the well-being of the animals. They were active, consumed normal amounts of food and water, fur remained sleek, and they did not lose weight. Data from 2 typical experiments are shown in Tables I and II. Ca and P concentrations maintained in the media surrounding bones from adrenalectomized animals were the same as controls (Table I).

In the cortisone-treated groups (Table II), while Ca concentrations remained unchanged, P concentrations were consistently lower than controls. The decrease in P concentration was marked after only 2 days of cortisone and became more pronounced with longer treatment. The Ca × P products were similarly reduced. Similar results were found with comparable doses of hydrocortisone.

Discussion. The fact that cortisone treatment induced a fall in P concentration, while adrenalectomy had no effect, suggests that the cortisone effect observed may have been the result of an excessive dose. However, although no significant effect on Ca concentration was noted, the changes in P which took place were in a direction which might have

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[§] This dose of cortisone is comparable to that used in experiments on rats by others(17). It was chosen for these experiments because preliminary experience indicated that it was effective in changing P distribution in the system employed and yet did not appear to affect the well-being of the animals nor cause them to lose weight.

[||] The media contained no Ca. P was present at a concentration of .40 mMol/1.

TABLE II. Effects of Cortisone.

		Ca	P	Ca × P
		mMol/l		
Controls	1	.64	.90	.58
	2	.64	.91	.58
	3	.60	.94	.56
	Mean	.63	.92	.57
Cortisone, 2.5 mg/day				
For 2 days	4	.62	.82	.51
	5	.67	.81	.54
	6	.63	.77	.49
	Mean	.64	.80	.51
For 5 days	7	.60	.74	.44
	8	.62	.80	.50
	Mean	.61	.77	.47
For 8 days	9	.64	.79	.51
	10	.63	.73	.46
	11	.65	.75	.49
	Mean	.64	.76	.49

been expected on the basis of observations from some laboratories of serum P concentrations in patients and experimental animals under the influence of adrenal steroids(14, 15). Our results suggest that one of the mechanisms by which cortisone decreases serum P levels is by changing the equilibrium for this ion between bone mineral and the extracellular fluid. This mechanism would serve to enhance the hypophosphatemic effects of increased glycogen deposition in muscle suggested by Fenn and confirmed by Nichols(19).

The details of the mechanisms involved in distribution of P between bone sample and medium which are influenced by cortisone cannot be determined from available data. Whether they are related to changes in citrate metabolism or changes in permeability or synthesis of the organic matrix requires further investigation.

Why changes in Ca distribution could not be shown to accompany the changes in P is equally unclear. Species differences may have been responsible, or changes in Ca distribution previously noted(1-8) may have been largely the result of phenomena remote from the bone itself such as absorption, excretion, etc.

It is of interest to compare the present results with those obtained previously using exactly the same system(18). It was shown that Ca concentrations were significantly higher when active cellular metabolism was present and following prior treatment with parathyroid hormone. P concentrations, however, were unchanged. In the present study changes were seen in P concentrations, while Ca concentrations were not affected. These findings suggest that cortisone may modify the steady state Ca × P concentration product by a different mechanism than parathyroid hormone.

Summary. Bones from cortisone-treated and adrenalectomized mice were incubated *in vitro* and Ca and P in the medium measured after a steady state was reached. Compared to controls, no significant changes were seen in the adrenalectomized group. In the cortisone-treated group, while Ca concentrations were unchanged, P concentrations were depressed. It is therefore suggested that the depression of serum P levels with cortisone observed in rats and man may be in part a result of a change in distribution of P between bone mineral and extracellular fluid.

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Blood Volume Parameters of a Poikilothermal Animal in Hypo- and Hyperthermia.* (26900)

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With the current interest in blood volume changes in warm-blooded animals produced by alteration in body temperature (particularly hypothermia) it appears logical that these phenomena be investigated in a poikilothermal animal as a possible source of insight into the nature of physiologic adjustment of blood volume to changes in body temperatures. Several common cold-blooded laboratory animals were considered for this study but due to ease of maintenance, size, and phylogenetic "highness" the alligator is the animal of choice.

Methods. Animals used in this study were 66 American Alligators, *Alligator mississippiensis,* weighing 615-1300 g and measuring 66.5-82.0 cm in length. The animals were obtained from Louisiana, kept in a shallow tank without food, and used within 2 weeks of the time they arrived but not during the first 3 days. Approximately an equal number of males and females were used, and no significant difference between sexes was noted with regard to the parameters studied.

The alligators were anesthetized using 30 mg/kg sodium pentobarbital injected intraperitoneally; anesthesia administered approximately 1½ hours before the experiments were begun proved to be most satisfactory. Cr^{51} tagged red cells were used to determine cell volumes, and I^{131} tagged serum albumin (RISA) was used to measure plasma volumes. The red cells to be tagged were obtained from donor alligators early in the day and tagged according to the technic of Gray & Sterling(1). Donor alligators were used only once although some recovered from this relatively massive bleeding (approximately 18 cc). A suspension of 5 cc of washed, tagged red cells suspended in enough 0.9% saline to approximate the normal hematocrit was given to each animal in the Cr^{51} series immediately after removal of a like volume of their own blood. The tagged blood-cell suspension was washed in with 1 cc of saline and an additional 1 cc of saline was injected slowly during the experimental period (about one hour).

Both withdrawal and injection, as well as subsequent bleeding, were done through an indwelling cardiac needle introduced by puncture. An initial hematocrit determination was made for each animal using the first blood drawn from the heart; for this determination micro-technics were used. Hematocrits were also measured for donor alligators. These hematocrits are subsequently called venous hematocrits although they may represent mixed venous and arterial blood.

In the I^{131} series one cc of a solution of RISA appropriately diluted in 0.9% saline was injected after blood had been withdrawn for hematocrit determinations. The same washing-in technic was used as in the case

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of the Cr^{51} series. The anti-coagulant used in all cases was polyanhydromanuronic acid sulfate (Manuronate—Wyeth Laboratories). One half-hour, found by previous experiments, was allowed for mixing of the isotopes. Each experimental group consisted of 3 animals. At the close of the mixing period cloacal temperatures were ascertained. After this one animal was set aside to continue at room temperature (22.5°C-26.5°C-av. 24°C) until termination of the experiment 30 minutes later; one animal was enclosed in a warming blanket and warmed as rapidly as possible to a cloacal temperature of 35°C; and the third animal was packed in crushed ice and chilled to a cloacal temperature of 5°C. The intention was to reach the experimental temperatures in 30 minutes. The actual warming time ranged from 25-60 minutes, averaging 40 minutes. Animals were bled rapidly and pithed when the desired temperature was reached but not earlier than 30 minutes. The terminal temperature in 80% of the animals was 35°C, in 5%–36°C, and in 15%–37°C. Cooling required 20-60 minutes, averaging 36 minutes. These animals were killed when body temperature reached 5°C. The terminal temperatures were 5°C for 70% of the animals, 4°C for 25%, and 3°C for 5%. After killing, the alligators were dissected as rapidly as possible; the organs were blotted, weighed, and digested in nitric acid or KOH as was appropriate to the tagging technic used. Radioactivity was determined for blood samples and aliquots of the solutions of organs.

Considerable effort was made to keep trauma and blood loss minimal. A previous series of 42 animals(2) had been subjected to the open chest technic with relatively high blood losses, and precautions were taken to avoid excessive blood loss in this series. However, the results obtained in the 2 series of animals seem to be in substantial agreement. Data for each animal were corrected to a standard total body count of one million and a standard total body weight of 1000 g. Tables I and II include the principal data obtained; methods used in calculating specific blood volume parameters and level of significance of the data are included within the tables.

Results and discussion. The average plasma volume of alligators kept at room temperature is 60.1 cc with a standard error of ±3.88 cc per 1000 g; average cell volume for this same group of animals is 12.6 cc ± 0.34 cc per 1000 g. Thus total circulating blood volume is 72.7 cc/1000 g of body weight. The circulatory hematocrit,

$$\text{Circulatory hematocrit} = \frac{\text{cell volume}}{\text{cell volume} + \text{plasma volume}} \times 100,$$

TABLE I. Mean Measured and Calculated Blood Volume Parameters, with Standard Errors, for the Alligator.

Treatment group	PV, ml/kg	CV, ml/kg	VH, %	CH,* %	BVR cells†	BV_1,‡ ml/kg	BV_2,§ ml/kg	Residual plasma,‖ ml/kg	BVR plasma¶
A Room temp. 25°C	60.1 ± 3.88	12.6 ±.34	22.6 ±.64	17.3	.76	72.7	55.9	16.8	1.07
B Chilled 5°C	56.6 ± 4.11	12.4 ±.12	19.8 ±.69	17.9	.90	69.0	62.3	6.7	1.02
C Warmed 35°C	72.8 ± 3.77	12.6 ±.82	23.0 ±.52	14.8	.64	85.4	54.8	30.6	1.11

P values for differences between means

$PV_A - PV_B = 3.43$ not significant

$PV_A - PV_C = 12.68$ $<.05$

$PV_B - PV_C = 16.11$ $<.02$

$VH_A - VH_B = 2.47$ $<.02$

$VH_C - VH_A = .66$ not significant

$VH_C - VH_B = 3.13$ $<.01$

PV = Plasma vol
CV = Cell vol
VH = Venous hematocrit
CH = Circulatory hematocrit

* Circulatory hematocrit = CV/PV + CV × 100.
† BVR cells = Blood vol ratio cells = CH/VH.
‡ $BV_1 = CV + PV$.
§ $BV_2 = CV/VH$.
‖ Residual plasma = $BV_1 - BV_2$.
¶ BVR plasma = Blood vol ratio plasma = 100–CH/100–VH.

TABLE II. Cell and Plasma Volumes (ml/kg), with Standard Errors, for Organs of Alligators.

	Heart	Spleen	Liver	Lungs	Stomach	Intestine	Kidney	Total viscera	Muscle	Skin
A—Room temp.										
PV	.710 ±.049	.211 ±.015	2.973 ±.195	1.221 ±.041	1.114 ±.103	1.377 ±.122	.880 ±.095	8.486	1.121 ±.163	1.417 ±.181
CV	.097 ±.017	.089 ±.023	.301 ±.017	.326 ±.022	.193 ±.021	.237 ±.028	.110 ±.005	1.353	.286 ±.022	.292 ±.026
Hematocrit	12.0%	29.8%	9.2%	21.1%	14.7%	14.6%	11.1%	13.7%	20.3%	17.0%
B—Cold										
PV	.677 ±.027	.266 ±.012	2.870 ±.161	1.237 ±.065	1.057 ±.056	1.334 ±.090	1.010 ±.085	8.451	.841 ±.043	1.406 ±.075
CV	.089 ±.007	.399 ±.037	.417 ±.022	.323 ±.016	.152 ±.011	.234 ±.011	.101 ±.007	1.715	.233 ±.014	.281 ±.027
Hematocrit	11.5%	60.0%	12.6%	20.7%	12.6%	14.9%	9.1%	16.8%	21.6%	16.6%
C—Warm										
PV	.821 ±.055	.243 ±.011	3.943 ±.274	1.866 ±.190	1.190 ±.133	1.952 ±.129	1.302 ±.138	11.317	1.219 ±.104	2.886 ±.296
CV	.060 ±.005	.070 ±.014	.307 ±.046	.337 ±.029	.146 ±.021	.141 ±.016	.076 ±.011	1.137	.261 ±.036	.292 ±.024
Hematocrit	6.8%	22.4%	7.2%	15.3%	10.9%	6.7%	5.4%	9.1%	17.6%	9.1%
					P values for differences between means					
$PV_A - PV_B$	—	—	—	—	—	—	—		—	—
$PV_C - PV_A$	—	—	<.05	<.01	—	<.01	<.05		—	<.01
$PV_C - PV_B$	<.05	—	<.01	<.01	—	<.01	—		<.01	<.001
$CV_B - CV_A$	—	<.001	<.01	—	—	—	—		—	—
$CV_A - CV_C$	—	—	—	—	—	<.05	<.05		—	—
$CV_B - CV_C$	—	<.001	<.05	—	—	<.001	—		—	—

PV = Plasma vol CV = Cell vol

is therefore 17.3 compared with a venous hematocrit of 22.5 ± 0.64. However, if blood volume is calculated from cell volume and venous hematocrit, it is only 55.9/1000 g giving a residual plasma volume(3) of 16.8 cc/1000 g not in the venous circulation but retained in the organs. This viewpoint is reinforced by the data on organ hematocrits, which, with the exception of the spleen, are lower than the venous hematocrits, most of them significantly so; skin and muscle hematocrits are also lower than venous hematocrit. Only the spleen, lungs, and muscles have hematocrits higher than circulatory hematocrit.

Comparing these data from a cold-blooded animal with those from a warm-blooded animal, the dog, shows several differences. The dog's plasma volume was 9.9/1000 g of tissue less than that of the alligator. The difference is significant at the <.01 level. Venous and circulatory hematocrits for dogs are higher than those of alligators; Deavers *et al.*(4) reported a venous hematocrit of 45.2% ± 0.73 and a circulatory hematocrit of 40.2% with a BVR cells ratio of 0.89.

$$\text{BVR cells} = \frac{\text{circulatory hematocrit}}{\text{venous hematocrit}}$$

For alligators this same ratio is 0.76. Finally total circulating blood volume is higher in dogs, 88.7 cc/1000 g, *i.e.*, 16.0 cc/1000 g greater than that of the alligator.

When alligators are cooled rapidly to a body temperature of 5°C there is usually a drop in venous hematocrit and the difference between the average hematocrit of these animals (19.8 ± 0.69) and of those kept at room temperature for the same length of time is 2.7%. This is in contradistinction to the reaction of the dog where cooling raises the hematocrit. Average plasma volume (56.6 ± 0.12), however, remains relatively constant; and therefore the circulatory hematocrit is not affected significantly, being 17.9%. Total visceral plasma volume (the sum of volumes for the 7 organs studied) for the cooled group remains the same and total visceral cell volume increases slightly; however, the only statistically significant difference is the increase in cell volume of the spleen. The change in venous hematocrit could be explained if there were a "trapping" or sequestering of red cells by the spleen and liver, and to some extent by a fall in total muscle plasma volume. Total residual plasma volume, however, decreases from an initial volume of 16.8 cc to a level of 6.71 cc/1000 g in cooled alligators. Other changes must be postulated in addition to the cell volume changes in spleen and liver to explain the magnitude of the fall in venous hematocrit; possibly the "lost" plasma of the muscles is added to the venous circulation.

Rapid warming of alligators to 35°C, approximately 10°C higher than the temperature to which they had been acclimated, caused no significant change in venous hematocrit (22.9 ± 0.52) although there was an upward trend in the hematocrits. There was a significant increase in total plasma volume to 72.8 ± 3.77 cc/1000 g but cell volume remained constant. As a result, the circulatory hematocrit fell to 14.75 and the volume residual plasma increased to 30.5/1000 g. Significant increases in organ plasma volumes were noted in the liver, lungs, intestine, kidney, and skin. Significant decreases in cell volumes were noted for intestine and kidneys, and, in addition, total visceral cell volume fell. Thus, total visceral plasma volume increased, while total visceral cell volume decreased. The venous hematocrit, therefore, may increase sightly in a majority of normal animals in spite of widespread increases in plasma volume because of release of red cells from viscera, particularly the intestines and kidneys. At the same time circulatory hematocrit falls because of the increases in plasma volume presumably coming from fluid released by extra-circulatory sources into the small vessels of the viscera, skin and possibly the muscles, but more specifically into the liver, lungs, intestines, kidneys, and skin.

Summary. Cooling the alligator rapidly to 5°C results in a lowered venous hematocrit but does not cause significant change in circulatory hematocrit. Total visceral cell volume tends to increase, although of the individual organs only one, the spleen, has a statistically significant increase in cell volume. Rapid warming of alligators to temperature of 35°C results in no significant change in venous hematocrit but in a significant fall in circulatory hematocrit. Organ cell volumes in general are lower, that of the intestine and kidneys significantly so. This release of cells must account for the maintenance of a constant venous hematocrit in spite of a great increase in plasma volume not accounted for solely by the significant increases in organ plasma volumes.

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Antigenic Properties of Liver Cell Fractions Derived from Laying Hens and Estrogenized Chickens. (26901)

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It is known that there are phosphoproteins in the sera of laying hens and estrogenized chickens, but not in the sera of non-laying hens, cocks and immature chickens, and that they are produced in the liver by the effects of estrogens(1,2,3,4). The fluctation of phosphoprotein level and of its properties in the sera of laying hens has been studied serologically, chemically and isotopically (5,6,7,8).

Recently, serum albumin and globulin syntheses *in vitro* have been performed by liver slices. Peter *et al.*(9) developed an immunological method by which the small amounts of serum albumin present in liver slices could be determined and showed that the liver was locus of serum albumin production. Higashi (10) also studied the production of serum globulin *in vitro,* using liver slices and their homogenates. There is no known report on phosphoprotein synthesis *in vitro* by chicken liver slices. Direct demonstration of protein syntheses *in vitro* is complicated by the fact that total protein content of tissue slices decreases upon incubation, owing to the predominance of proteolytic reactions. Our experiments are intended to clarify the mechanism of phosphoprotein synthesis in liver cells of the chicken, and to study the antigenic properties of liver cell fractions of laying hens and estrogenized immature chickens.

Materials and methods. Chickens used for fractionations of liver cell components were White Leghorn laying hens, cocks, and 30-day-old chickens injected with 0.625 mg of diethylstilbestrol intramuscularly every day for 5 days. After serologically demonstrating the presence of, and determining level of phosphoproteins in their sera, the chickens were sacrificed by bleeding in a cool room at 4°C. The liver was perfused with 0.15 M NaCl solution and removed for fractionation of liver cell components. The method of Hogeboom(11) was used for fractionation. Ten ml of liver tissue was sliced, washed with 0.15 M NaCl and homogenized (20% homogenate with 0.25 M sucrose solution). Nuclear, mitochondrial and microsomal fractions were washed by centrifugation twice with 0.34 M sucrose solution and once with distilled water. Each fraction was suspended in 10 ml of distilled water and used for immunization. A similar fractionation technic was used to get spleen cell components from estrogenized chickens. Fractions of spleen cells and of cock sera were dried by freeze-drying before using them in the absorption of antibodies, subsequently called DCS (Dried Cock Serum). The supernatant fraction was diluted with 10 volumes of distilled water and kept at room temperature for 2 days. The precipitate thus obtained was used for absorption. Immune sera were obtained by injecting rabbits intravenously with 2 ml of each suspension every other day 4 to 5 times, and 7 days after the last injection, animals were bled from their jugular arteries. Antisera were inactivated by keeping them in a water bath at 56°C for 30 minutes and 0.1% by volume of a Na-azide solution was added. S-I and S-III fractions of laying hen serum were prepared by the ultracentrifugation technic described by Tanabe *et al.*(12). The technic of interfacial precipitin reaction was described earlier(4). The following technic (Oudin) was used for the agar gel diffusion reaction: Antiserum was mixed with the same volume of 1% agar gel solution at 56°C and poured at once into test tubes of 3 mm diameter to a height of about 2 cm. After coagulation of the agar gel, about the same volume of test serum was layered on the gel, then covered with liquid paraffin to prevent evaporation of the test serum. The test tubes were kept at room temperature (10-18°C) for 4 days. The reactions were read by naked eye. Since 60 mg of DCS was equivalent to a net weight of 1 ml cock serum, 90 mg of DCS were added to each ml of immune serum to exhaust the species specific antibodies. Besides the antisera shown in Table I, all antisera used were absorbed with DCS to exhaust the species specific antibodies. Ten mg of a microsome fraction prepared by freeze-drying from an equivalent of net weight of 5 g of liver tissue, or precipitates taken from 15 ml of supernatant fraction, were added to 0.5 ml of antisera to test the absorbing properties of their antibodies. Titration of antibodies was carried out by the precipitin reaction, serially diluting antisera with 10% NaCl solution.

Results. The results of serological precipitin reactions obtained by antisera against liver and spleen cell fractions of laying hens are shown in Table I.

All unabsorbed antisera showed positive reactions to sera of laying hens and cocks.

TABLE I. Precipitin Reactions of Antisera Obtained with Liver and Spleen Cell Fractions of Laying Hens.

		Antisera										
		Unabsorbed						Absorbed with DCS				
		Dilution of antigen										
Antisera obtained with fractions of	Antigen	20	40	80	160	320	640	20	40	80	160	320
Liver nucleus	Cock	3	2	1	0	0	0	0	0	0	0	0
	Hen	3	3	3	3	2	0	0	0	0	0	0
Mitochondria	Cock	2	2	0	0	0	0	0	0	0	0	0
	Hen	3	3	3	3	1	0	0	0	0	0	0
Microsome	Cock	3	3	2	2	0	0	0	0	0	0	0
	Hen	3	3	3	3	3	3	3	3	3	3	1
Supernatant	Cock	3	3	3	3	0	0	0	0	0	0	0
	Hen	3	3	3	3	3	3	2	2	2	1	0
Spleen microsome	Cock	2	2	2	2	2	0	0	0	0	0	0
	Hen	2	2	2	2	1	1	0	0	0	0	0
Supernatant	Cock	3	3	3	3	2	2	0	0	0	0	0
	Hen	3	3	3	3	1	1	0	0	0	0	0

3, 2 and 1 show the appearance of positive reaction within 15, 30 and 60 min., respectively; 0 shows no reaction.

DCS = Dried cock serum.

After absorption of antisera with DCS, each antiserum against the microsomal and supernatant fractions of laying hens showed good reactions to laying hen serum, but not to cock serum. However, antisera against microsomal and supernatant fractions of spleen cells and nuclear and mitochondrial fractions of liver cells from laying hens did not show any positive reaction for presence of antigenic properties of phosphoprotein. No antisera were produced against phosphoproteins in sera of laying hens by liver cell fractions of cocks. These results were confirmed in 3 experiments with microsomal and supernatant fractions of laying hens.

Similar experiments were carried out to test for presence or absence of antigenic properties of phosphoprotein in microsomal and supernatant fractions from liver cells of estrogenized or normal immature chickens. The results were similar to those shown for the antigenic properties of microsomal and supernatant fractions of estrogenized immature chickens. No reactions were produced by other fractions from the same animals and fractions of liver cells of untreated immature chickens.

Table II summarizes absorption tests of anti-Mc(lay) and anti-Sup(lay) with S-I and S-III fractions of laying hens, respectively.

Before absorption of antisera, they showed positive reactions with both fractions of S-I and S-III. After absorbing anti-Mc(lay) with S-I, however, the antiserum showed no reaction with S-I, but reacted positively with

TABLE II. Absorption Tests of Antisera with S-I and S-III Fractions of Laying Hens.

		Dilution of antigen											
		S-I						S-III					
Antisera	Absorbed with	20	40	80	160	320	640	20	40	80	160	320	640
Anti-Mc(lay)	Unabsorbed	1	1	1	1	0	0	3	3	3	2	2	0
	S-I	0	0	0	0	0	0	3	3	3	1	0	0
	S-III	0	0	0	0	0	0	0	0	0	0	0	0
Anti-Sup(lay)	Unabsorbed	3	3	3	3	2	0	3	3	3	3	3	1
	S-I	0	0	0	0	0	0	3	3	3	3	1	0
	S-III	3	2	1	1	0	0	0	0	0	0	0	0

Mc(lay) and Sup(lay) are microsome and supernatant fractions derived from liver cells of laying hens, respectively.

TABLE III. Decreases of Antibody Titers by Absorption with Microsome and Supernatant Fractions.

Dilution of antisera	Antigen	Anti-Mc(lay)																			
		Unabsorbed					Absorbed with														
							Mc(lay)					Mc(cock)					Mc(lay-spleen)				
		Dilution of antigen																			
		20	40	80	160	320	20	40	80	160	320	20	40	80	160	320	20	40	80	160	320
1	S-III	3	3	3	3	2	0	1	1	1	0	3	3	3	3	2	3	3	3	3	2
2		3	3	3	3	2	0	0	0	0	0	3	3	3	3	2	3	3	3	3	2
4		1	2	2	1	1	0	0	0	0	0	1	2	2	1	0	1	2	2	1	0
8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Anti-Sup(lay)																			
		Unabsorbed					Absorbed with														
							Sup(lay)					Sup(cock)					Sup(lay-spleen)				
1	S-I	3	3	3	3	2	0	0	0	0	0	3	3	3	3	2	3	3	3	3	2
2		3	3	3	3	2	0	0	0	0	0	3	3	3	3	2	3	3	3	3	2
4		3	2	2	2	0	0	0	0	0	0	2	2	1	1	0	3	2	2	1	0
8		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Mc(lay-spleen) and Sup(lay-spleen) are microsome and supernatant fractions derived from spleen cells of laying hens, respectively.

S-III. Absorption of the antiserum with S-III fraction exhausted antibodies to S-I and S-III fractions. Absorption tests of anti-Sup(lay) with S-I and S-III fractions showed that S-I and S-III fractions removed antibodies for themselves from the antiserum, but did not remove antibodies for other fractions.

In the following experiment, titration of antibodies in anti-Mc(lay) and anti-Sup (lay) absorbed with microsomal and supernatant fractions of liver and spleen cells of laying hens and of liver cells of cocks, respectively, was carried out (Table III).

Antibody to S-III in the anti-Mc(lay) was almost removed by absorption with Mc(lay). But no decrease of antibody occurred by absorption with Mc(cock) and Mc(lay-spleen), respectively. Similarly, antibody to S-I fraction in the anti-Sup(lay) was exhausted by absorption with Sup(lay), but not by absorption with Sup(cock) and Sup(lay-spleen).

Antisera to each fraction of liver cells of laying hens were used for agar gel diffusion reaction with sera of laying hens and cocks and with S-I and S-III fractions. In these experiments, one positive zone reaction appeared in the agar gel of anti-Mc(lay) with serum and S-III fraction of laying hens and 2 reactions in the agar gel of anti-Sup(lay) with serum and S-I fraction of laying hen. Absence of positive zone reaction to S-I fraction of laying hen in the agar gel of anti-Mc(lay) is assumed to be due to a deficiency of antibody against the S-I fraction of laying hen. Thus, the results obtained here by agar gel diffusion reaction demonstrated again the presence of the specific antigenic properties of S-I and S-III fractions of laying hen serum in microsomal and supernatant fractions of liver cells of laying hens and estrogenized immature chickens.

Discussion. It has been known that the liver is the locus of serum albumin and globulin production in mammals and that the microsomal fraction plays an important role in protein synthesis in liver cells(13,14). In our experiments, microsomal and supernatant fractions of liver cells of laying hens and of estrogenized chickens showed immunologically antigenic properties equivalent to phosphoproteins present in the sera of laying hens. However, fractions of liver cells of cocks and immature chickens and of spleen cells of estrogenized chickens had no such antigenic properties. The absence of specific antibodies in the antisera produced by immunizing with nuclear and mitochondrial fractions of liver cells and microsomal and supernatant fractions of spleen cells derived from laying hens and estrogenized chickens suggests that the antigenic properties of phosphoproteins found in the 2 fractions of liver cells of laying hens were not those induced by residual serum proteins in liver homogenates. The antigenic properties of phosphoproteins found in microsomal and supernatant fractions of laying hens and estrogenized chickens appear to be specific for the 2 fractions of their liver cells. These findings give immunological support to the idea that the liver is the locus of phosphoprotein production in the laying hen. Microsomal and supernatant fractions of liver cells of laying hens had both specific antigenic properties of S-I and S-III, in which antigenicity of S-I in the microsomal fraction was weak. The reason for this weakness is not clear. The results permit the assumption that microsomes play an important role in phosphoprotein syntheses in liver cells or that they produce the S-I fraction (low density lipoprotein) and S-III fraction (phosphoprotein) in liver cells of laying hens and secrete them into blood stream.

Summary. Microsomal and supernatant fractions of liver cells of laying hens and estrogenized chickens showed immunologically specific antigenic properties similar to those of phosphoproteins found in the sera of laying hens. However, nuclear and mitochondrial fractions derived from liver cells of laying hens, 4 fractions from spleen cells of laying hens and 4 fractions from liver cells of cocks, did not show any such specific antigenic properties. Microsomal and supernatant fractions of liver cells of laying hens and estrogenized chickens had both specific properties of S-I and S-III fractions. Microsomal and supernatant fractions absorbed well anti-S-III and anti-S-I antibodies, respectively.

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Elevation of Proteolytic Activity in the Pancreas of Hypophysectomized Rats by Hormonal Therapy.* (26902)

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Following hypophysectomy a severe reduction in weight of the pancreas occurs in the rat(1) and dog(2). In the rat most acini are partially involuted with loss of cellular size, zymogenic granules and cytoplasmic RNA(3,4,5). Concurrently, the amount of amylase(6) and proteolytic activity(7) is reduced. Experiments aimed at clarifying the hormonal pathway by which the hypophysis exerts its influence over the pancreas have entailed both ablation of other endocrine glands and hormonal replacement therapy of endocrine-deficient animals. These studies reveal that thyroid hormone plays a key role(3,8,9,10,11,12) in maintaining the

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normal structure of pancreatic acini. Hypophyseal somatotropin(13,14) and adrenocorticosteroids(15,16,17,18) are also implicated. Indeed, administration of combined somatotropin, corticosterone and l-thyroxine to *ad libitum*-fed hypophysectomized rats restores pancreatic weight and histology to an approximately normal condition(19).

Few attempts have been made to restore to normal the content of digestive enzymes in the pancreas of hypophysectomized animals by hormonal therapy. Nishikawara *et al.*(20) found feeding of thyroid tissue to be effective with respect to amylase. This report is concerned with the capacity of combined administration of somatotropin, corticosterone and l-thyroxine (STC) to elevate proteolytic activity (PA) in the pancreas of hypophysectomized rats.

Procedures. Young adult, female Sprague-Dawley rats were fed *ad libitum* a diet consisting of Purina Laboratory Chow supplemented with greens and cod liver oil. After hypophysectomy, a mean of 16 to 25 days elapsed before initiation of hormonal therapy (Table I). The rats were divided into the following groups: (a) hypophysectomized, vehicle - treated, (b) hypophysectomized, hormone-treated, and (c) nonhypophysectomized, vehicle-treated. Somatotropin† was dissolved in distilled water (1.25 mg/ml); corticosterone was suspended in 0.9% NaCl (150 μg/ml) with one drop of Tween 80 being added/10 ml of saline; and sodium l-thyroxine pentahydrate was dissolved in distilled water (60 μg/ml) with one drop of N/10 NaOH per 50 ml of solution being used to facilitate solution of the hormone. Twice daily, 125 μg of the somatotropin, 15 μg of corticosterone and 3 μg of thyroxine were administered together in one injection. One-half unit of insulin was added in Experiment 1 to the treatment of Group 3. Nonhypophysectomized rats received an equal volume of 0.9% NaCl, with Tween 80 and N/10 NaOH being added in the amounts used for dissolving the hormones. Treatment was continued for 14 to 43 days at which time food, but not water, was denied the animals for 24 hours in Exp. 1 and 18 hours in Exp. 2 and 3 prior to termination of experiment. The pancreas was removed while the rats were under sodium amytal anesthesia and dissected free of extraneous fat and connective tissue. Completeness of hypophysectomy was verified by microscopic examination of sections of the pituitary area in all hypophysectomized rats treated with hormones and in the hypophysectomized, vehicle-treated rats which exhibited a questionable loss in body weight.

The procedure for determination of PA (activated trypsinogen, chymotrypsinogen) was described previously(7), and is based on release from a substrate containing hemoglobin(21) of tyrosine-like compounds by pancreatic proteolytic enzymes. The color developed following addition of the Folin-Ciocalteu reagent was determined in a Klett-Summerson colorimeter and PA expressed as mg of tyrosine released per minute at 37°C by reference to a standard curve previously worked out with the pure amino acid. Our previously published procedure was altered in the following ways. The original pancreatic homogenate was diluted to a final concentration of 1 g of pancreas in 1.25 L of physiological saline. The homogenate was brought to pH 5 by addition of 0.1 M phosphate buffer in Exp. 1 and 2 and by addition of acetate buffer in Exp. 3. Five ml aliquots of the homogenate were activated by addition of 0.1 ml (10 μg) of trypsin and warming at 37°C for 24 hours. Trypsin was found to effect complete activation much more consistently than was true of the enterokinase. Nitrogen content of the homogenate was determined by the micro-Kjeldahl method(22) and expressed in relation to wet weight of the gland.

Results. Treatment with STC increased the body weight of hypophysectomized rats to a level comparable with that of the nonhypophysectomized controls (Table I). Similarly, the weight of the pancreas was in-

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TABLE I. Data on Treatment and Body Weights.

Group	Treatment	No. of rats	Mean days hyp. to treatment	Mean days of treatment	Mean body wt (g)			Mean wt pancreas (mg)
					At hyp.	At inj.	Final	
Exp. 1								
1	Hyp.–veh.	11	24 ± 2*	29 ± 3	174 ± 6	159 ± 3	164 ± 3	516 ± 35
2	Hyp.–STC	9	27 ± 3	31 ± 4	174 ± 5	157 ± 6	239 ± 7	920 ± 43
3	Hyp.–STC–ins.	3	25 ± 5	29 ± 1	188 ± 5	164 ± 6	250 ± 9	1052 ± 19
4	Nonhyp.–veh.	13	—	—	182 ± 4	212 ± 4	240 ± 5	1128 ± 44
							1 *vs* 2 <.001†	1 *vs* 2 <.001
							2 *vs* 4 >.05	2 *vs* 4 <.01
							1 *vs* 4 <.001	1 *vs* 4 <.001
Exp. 2								
5	Hyp.–veh.	4	16 ± 0	21 ± 2	197 ± 3	175 ± 1	174 ± 2	603 ± 21
6	Hyp.–STC	5	16 ± 0	21 ± 2	197 ± 2	183 ± 3	235 ± 1	924 ± 22
7	Nonhyp.–veh.	5	—	—	198 ± 2	214 ± 2	229 ± 4	1049 ± 43
								5 *vs* 6 <.001
								6 *vs* 7 >.05
								5 *vs* 7 <.001
Exp. 3								
8	Hyp.–veh.	13	22 ± 1	17 ± 1	172 ± 3	153 ± 3	150 ± 2	454 ± 23
9	Hyp.–STC	14	22 ± 1	17 ± 1	172 ± 2	148 ± 3	215 ± 2	840 ± 18
10	Nonhyp.–veh.	14	—	—	170 ± 1	200 ± 2	214 ± 3	930 ± 23
							8 *vs* 9 <.001	8 *vs* 9 <.001
							9 *vs* 10 >.05	9 *vs* 10 <.01
							8 *vs* 10 <.001	8 *vs* 10 <.001

* Stand. error of mean. † Student Fisher "t"; probability of error.

Hyp. = hypophysectomized; STC = somatotropin, corticosterone, thyroxine; veh. = vehicle; ins. = insulin.

creased in hypophysectomized rats but not to non-operated control levels.

The amount of nitrogen per g of pancreas (Table II) was reduced by hypophysectomy in Exp. 3; in Exp. 2 change in the same direction was indicated. Hormone therapy failed to alter the concentration of nitrogen. However, total nitrogen content was reduced by hypophysectomy and increased by hormone treatment.

Total PA was reduced by hypophysectomy and increased significantly by STC over that in hypophysectomized rats although not up to the level observed in nonhypophysectomized rats (Exp. 1 and 3). However, the influence of hypophysectomy and of treatment with STC on concentration of PA varied from experiment to experiment. PA per g of pancreas was reduced by hypophysectomy in Exp. 1 and was increased by STC. When related to nitrogen per g of pancreas, PA was increased by hypophysectomy, these values being restored to the level of those in nonhypophysectomized rats by hormone therapy. This trend was also evident in Exp. 2 and 3 when PA was related to the wet weight of the pancreas.

Addition of insulin (Exp. 1) to the hormone therapy appeared to increase the effectiveness of the treatment in every respect.

Discussion. In a previous study, in which the pancreatic homogenate was activated with enterokinase(7) a reduction in concentration of PA was demonstrated consistently after hypophysectomy, although with the exception of those animals studied 3 days after operation, this decline was not great. Haist(23) observed variable changes in PA after pituitary ablation. In the present study, a reduction was observed only in Exp. 1 when the significance of the difference between the activity in hypophysectomized and nonhypophysectomized rats appeared to arise chiefly from the high values for the latter group. The high level in the controls probably occurred because these animals were deprived of food for a longer time before being killed than was true for the rats in Exp. 2 and 3. Thus, a greater build-up of stored enzyme was permitted in the nonhypophysectomized rats.

The response of pancreatic PA to hypophysectomy differs somewhat from that of amylase. Although total content of both is low-

TABLE II. Effect of Hormone Treatment on Proteolytic Activity and Nitrogen.

Group	Treatment	Nitrogen (mg/g pancreas)	Total nitrogen (mg)	Proteolytic activity (mg tyrosine)		
				Per g pancreas	Total	Per mg nitrogen* × 100
Exp. 1						
1	Hyp.–veh.			8.8 ± .5†	4.6 ± .5	
2	Hyp.–STC			11.6 ± .8	10.6 ± .8	
3	Hyp.–STC–ins.			13.3 ± 1.6	14.1 ± 2.0	
4	Nonhyp.–veh.			10.8 ± .5	12.3 ± .8	
	1 *vs* 2			<.01‡	<.001	
	2 *vs* 4			>.05	>.05	
	1 *vs* 4			<.01	<.001	
Exp. 2						
5	Hyp.–veh.	34.4 ± 1.0	20.8 ± 1.0	10.9 ± 1.2	6.5 ± 1.0	31.4 ± .3
6	Hyp.–STC	36.7 ± 1.2	34.4 ± 1.6	8.6 ± 2.0	7.9 ± .7	23.4 ± 1.6
7	Nonhyp.–veh.	36.2 ± 1.2	37.7 ± 1.8	8.4 ± .6	8.7 ± .7	23.1 ± 1.4
	5 *vs* 6	>.05	<.001	>.05	>.05	>.05
	6 *vs* 7	>.05	>.05	>.05	>.05	>.05
	5 *vs* 7	>.05	<.001	>.05	>.05	=.05
Exp. 3						
8	Hyp.–veh.	30.6 ± .8	13.8 ± .8	8.4 ± .4	3.8 ± .3	27.5 ± 1.4
9	Hyp.–STC	30.5 ± .8	25.5 ± .8	6.9 ± .5	5.8 ± .5	22.1 ± 1.7
10	Nonhyp.–veh.	34.0 ± .7	31.6 ± 1.1	7.3 ± .5	6.9 ± .5	21.1 ± 1.3
	8 *vs* 9	>.05	<.001	<.05	<.01	<.05
	9 *vs* 10	<.01	<.001	>.05	>.05	>.05
	8 *vs* 10	<.01	<.001	>.05	<.001	<.01

* $\frac{\text{mg tyrosine/g pancreas}}{\text{mg nitrogen/g pancreas}} \times 100.$

† Stand. error of mean.

‡ Student Fisher "t"; probability of error.

Hyp. = hypophysectomized; STC = somatotropin, corticosterone, thyroxine; veh. = vehicle; ins. = insulin.

ered, the concentration of only amylase is reduced(6). Correlated with this evidence of depressed protein synthesis are the depletion of RNA as revealed by basic staining(3) and biochemical analysis(5) and the reduced quantity of zymogenic granules(4).

It is clear that a large measure of restoration of total PA can be effected by administration of somatotropin, corticosterone and thyroxine if the period of treatment is sufficiently prolonged (Exp. 1). Addition of insulin facilitates the effect. It is probable that other hormones, especially from the hypophysis, would augment the restoration further.

Summary. Hypophysectomy of the rat failed to reduce consistently the concentration of PA in the pancreas when computed on the basis of wet weight. When related to mg of nitrogen per g of pancreas the amount of PA was increased. Treatment with STC restored body weight, increased pancreatic weight and total PA. Except when insulin was added to the hormonal regimen, PA did not reach the level observed in the nonhypophysectomized controls.

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Predictability of the Changes in Hematocrit which Follow Repeated Withdrawal of Blood.* (26903)

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A formula was derived for determining hematocrits (Hcts) of successive blood samples on the basis that sampling losses were being exactly replaced by extravascular fluid. When applied to experimental data, good agreement between measured and theoretical Hcts were encountered in several series of tests, as described herein.

Procedure. With the assumption that total blood volume remains constant through replacement of lost cells and plasma by extravascular fluid, it can be shown that the hematocrit of any particular blood sample (Hct_n) in a consecutive series of samples of constant size, is given by the exponential equation:

$$\mathrm{Hct}_n = \mathrm{Hct}_o \left(1 - \frac{s}{\mathrm{BV}}\right)^{n-1}$$

Where:

Hct_o = hematocrit of the initial sample in a series
s = sample size (constant over the series)
BV = total blood volume
n = number of blood samples taken

Hematocrits so calculated were compared to Hcts measured during experiments which required repeated handling and multiple blood samplings in the chicken.

All studies were conducted on unanesthetized adult male chickens of the White Leghorn breed, 12-24 months of age. Hematocrits were measured on heparinized blood samples centrifuged in graduated tubes for 20 minutes at approximately 1500 × g. As the blood volume (BV) required by the formula had not been determined on the birds for which Hcts were available, and as literature values for BV of adult male chickens varies from 9-10%(1) to 5.6 ml/100 g body weight(2), it was necessary to measure this parameter in a group of comparable animals. The method used was basically the T-1824 dilution technic(3), except that the calculations were made on a single sample of wing vein blood taken 2 min. after injection of dye.

Results and discussion. Blood volume. Total BV of 8 chickens weighing 2491 ± 48 g (SE) was determined to be 6.6 ± 0.25 ml (SE) per 100 g body weight (BW), and

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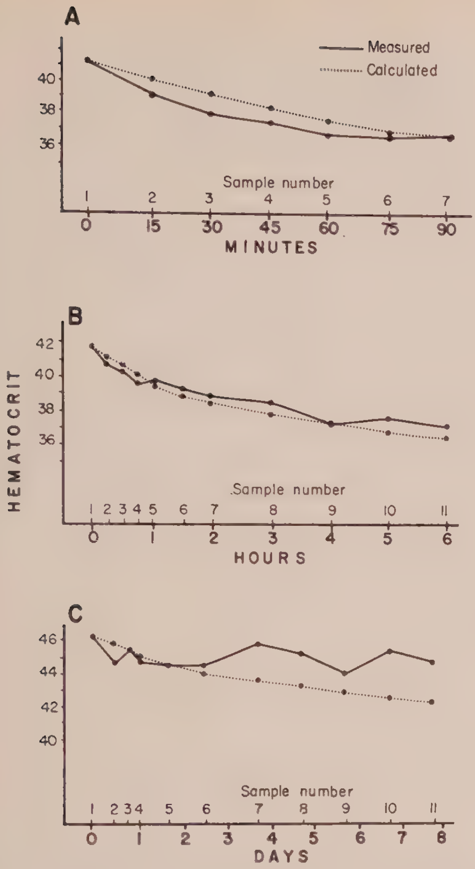

FIG. 1. Measured and calculated hematocrits following repetitive blood sampling in adult, male, White Leghorn chickens. Sample size: A—3½ ml, B—2½ ml, C—1½ ml.

this value was used to estimate the BV required in the formula. While this value for BV is close to the 5.6 ml/100 g BW found by Bond and Gilbert for White Leghorns, it should be noted that it is considerably lower than the 9-10% of BW suggested by Newell and Shaffner(1) for New Hampshire males of similar BW. Part of this discrepancy may well be due to breed differences, but it is also likely that the single 10-min. post-injection sample used by Newell and Shaffner (1) resulted in an overestimate of BV because of the rapid disappearance rate of T-1824 in the chicken(4).

Short term studies. Fig. 1-A shows the calculated and measured Hcts in a trial in which 3½ ml of blood was removed at 15-min. intervals within a 2-hr period. The 9 birds used averaged 2345 ± 88 g in BW and Hct_o averaged 41.2 ± 1.2 (SE). At the end of the 7th sample the animals had lost approximately 16% of their BV. Measured and calculated Hcts decreased in a parallel manner in this series, although the measured values are generally lower. However, with an average difference at each sampling of 0.7 ± 0.7 Hct unit, the two sets are within experimental error, and the calculated Hcts estimated measured Hcts with an average discrepancy of approximately 2%. It is conceivable that had BV been measured separately on each of these birds, both the bias and the error could have been reduced.

In a second series (Fig. 1-B) 2½ ml of blood were taken first at 15 min., then at 30 min., and finally at 60 min. intervals over a 6-hour period. The BW of the 7 birds used averaged 2626 ± 104 g, and Hct_o 41.8 ± 2.4. By the 11th sample, some 16% of BV had been removed. The parallel trend of both measured and calculated Hcts toward lower values is clearly evident. No statistical significance attaches to the average difference of 0.5 ± 1.0 between the 2 sets at each sampling, and the average error in estimating the Hct here was approximately 1¼%.

Thus in trials where multiple, closely spaced blood samples are taken, and up to 16% of blood volume is removed, Hct can be predicted by the formula with an average error of 2% or less, even when using an estimated value for BV. Since the calculations are based on replacement of lost cells and plasma entirely by extravascular fluid, it appears that the chicken, like the dog(5), is capable of "rapid and delicate" readjustment and maintenance of BV. It follows further that the mechanism by which this is accomplished involves little or no addition of cells to the vascular compartment, a fact which is not in disagreement with the normal rate of replacement of RBC volume of less than 1% in 6 hours,(6), and the lack of function of the spleen as an erythrocyte reservoir in the chicken(7). Theoretically then, under these conditions significant differences between calculated and measured Hct could be interpreted to mean that the movement of extravascular fluid has not been able to maintain BV constant, the proportional changes in BV being estimated by the ratio: calculated Hct/measured Hct.

Long term study. By way of contrast with the previous 2- and 6-hour trials, Fig. 1-C depicts the Hcts observed in an 8-day series. The 10 birds here were on a feed deprivation test and had lost 21% of their initial 2326 ± 60 g BW before feed was restored on the 8th day. Sample size was 1½ ml, taken at 12, 18, and 24 hours, and daily thereafter. Initial Hct was 46.2 ± 1.0. Except for the period between 18 and 60 hours, there was little agreement between measured and calculated Hcts. At 12 hours, after a single 1½ ml sample, measured Hct was lower than expected, very suggestive of the hemodilution noted by Sturkie and Newman(3) under similar conditions. From the 3rd day on, measured Hcts were significantly larger than calculated values (pooled SE of differences = ± 0.8) and, in general, had returned close to the initial level. While this is highly suggestive of addition of cells to the vascular compartment, it must not be overlooked that inanition itself can raise Hct due to relative loss of plasma(8). In any event, the equation obviously cannot be used to predict Hcts under these conditions.

It should be noted that effects on Hct of such factors as size of tube, force of centrifugation, trapped plasma, and site of sampling should not alter the relationship between measured and calculated values as long as the test conditions remain uniform within any one series of measurements.

Summary. A formula has been derived for calculating the hematocrit of successive blood samples based on the assumption that blood volume is maintained constant through replacement of lost cells and plasma by extravascular fluid. The formula predicted, with an error of 2% or less, decreasing hematocrits which followed a removal of 7-11 successive blood samples of 2½-3½ ml each, within a period of 6 hours or less in the adult male chicken. The formula failed to predict the Hcts observed in an 8-day test on fasting chickens in which 1½ ml blood samples were obtained at 12-24-hour intervals. In the course of this study, the blood volume of the 2.5 kg White Leghorn male was determined to be 6.6 ± 0.25 ml/100 g body weight.

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Adaptation of Columbia SK Virus to Sarcoma 180 Ascites Tumor Cells *in vivo* and Passage in the Cells *in vitro*. (26904)

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Viruses growing in tissue culture provide a desirable means for testing the effects of antiviral compounds. Unfortunately, the Columbia SK virus has not in the past been successfully cultured serially in established cell cultures(1), although we have reported (2,3) that the virus could be adapted to Ehrlich ascites tumor cells *in vivo*. We now show that Columbia SK virus adapted to Ehrlich cells *in vivo* can also be adapted to Sarcoma 180 ascites tumor cells *in vivo* after serial passage in mixtures of the 2 cells, and that the new strain can propagate efficiently even in *in vitro* cultures of Sarcoma 180 ascites cells. Clear cytopathic effects (CPE) and high titers of hemagglutinins (HA) are produced. The new virus-host system is proving useful, in parallel with the usual studies in mice, to study the effect of the antiviral substance "propionin"(4).

Materials and methods. 1). *Virus passage* in vivo. The original virus source was our 43rd passage of Columbia SK virus in Ehrlich ascites tumor cells (E43) in mice. This had a potency of about 10^6 LD_{50}/0.1 ml and 1:32 HA just before use. Two-tenths ml of a 10^{-3} dilution of the E43 ascites fluid was added to a mixture of 0.2 ml of Ehrlich ascites cells and 0.2 ml of Sarcoma 180 ascites cells both freshly harvested from ascites tumor-carrying mice. The infected mixture was immediately inoculated into the peritoneal cavities of 3 to 5 mice. Before the mice died, at 3 to 5 days after inoculation, ascites fluid was aspirated and the supernatant after centrifugation was used for the next virus passage. Microscopic observation was also made, after Giemsa staining, for detection of oncolysis.

2). *Virus passage* in vitro. After washing, 2 to 4 × 10^6 Sarcoma 180 ascites cells harvested freshly from mice were suspended in 1 ml of culture medium per tube. The medium was composed of 80% of Medium 199 and 20% of S180 ascites serum from which the fibrin had been removed by standing at 4°C for 1 week. One-tenth ml of a definite dilution of source virus with Medium 199 was inoculated into each tube. The culture was stationary at 36°C. After 2 to 4 days incubation, the cultures were shaken by hand to remove the cells from the glass wall, and then centrifuged. The supernatant was used for the next passage, and also for titration of the infectivity and for HA. The cells were resuspended in saline and stained with 1 drop of 0.2% of erythrosin, which colors the damaged cells red but does not stain living cells. With this staining method, the CPE of the virus can be calculated quantitatively. For the HA test, 0.2% sheep red cells suspended in potassium barbital buffer were used(5). The infectivity of the culture fluids was determined in mice injected with Sarcoma 180 cells, and *in vitro* in culture tubes.

Results. 1). *Adaptation of Columbia SK virus from Ehrlich ascites cells* in vivo *to Sarcoma 180 ascites cells* in vivo. Several preliminary trials showed that the Ehrlich-adapted strain of virus could not propagate directly in Sarcoma 180 ascites cells. One of the trials is shown in the top 2 lines in Table I. Two-tenths ml of 10^{-3} dilution of the 43rd passage of Ehrlich-adapted strain of virus (E43), mixed with 0.2 ml of Sarcoma 180 ascites cells, resulted in the death of each of 4 mice in 4 to 5 days, without oncolysis. Then a 10^{-2} dilution of the infected ascites harvested just before death (named S_1) was again inoculated into mice with fresh S180 ascites cells by the same method, and the 4 mice died at 7 to 8 days without oncolysis. The harvest (S_2) was not infectious when 10^0 to 10^{-3} dilutions were inoculated along with S180 ascites cells into mice. This indicates that the Ehrlich-adapted virus cannot adapt to Sarcoma 180 cells directly.

In contrast to the results with S180 cells alone, a mixture of Ehrlich cells and S180 cells supported virus propagation; that is, E43 propagated in the mixture with severe oncolysis of both types of cells, and the harvest $(SE)_1$ showed a clear hemagglutination. This harvest $(SE)_1$ could be passaged serially in the mixtures of both cells but not in the S-180 cells alone. At each generation, $(SE)_1$, $(SE)_2$, $(SE)_3$ and $(SE)_4$, attempted passage to Sarcoma 180 ascites cells alone was unsuccessful as shown further in Table I. Unexpectedly, at the 5th generation, it was found that $(SE)_5$ could propagate in S180 ascites cells alone with a clear oncolysis, and the harvest $(SE)_5S_1$ showed a definite HA indicating the existence of mature virus of at least 10^6 LD_{50}. The following passages from $(SE)_5S_1$ grew easily in Sarcoma 180 ascites cells alone. Further passage *in vivo* was stopped after 5 more generations (S_5). The newly adapted strain also propagated in Ehrlich ascites cells alone.

2). *Virus passage* in vitro. The *in vivo* harvest $(SE)_5S_2$ was centrifuged and 0.1 ml of 10^{-1} dilution of the supernatant was inoculated into culture tubes which contained Sarcoma 180 ascites cells. After 3 days of culture complete CPE had appeared, and the

TABLE I. Adaptation of Columbia SK Virus from Ehrlich Ascites Tumor Cells *In Vivo* to Sarcoma 180 Ascites Tumor Cells *In Vivo*.

					Harvests	
Inoculum	Dilution	Cells	Oncolysis	Day mouse died	No.	HA titer
E43	10^{-3}	Ehrlich	+	4, 4, 4	E44	64
E43	10^{-3}	S180	—	4, 5, 5, 5	S_1	0
E43	10^{-3}	Ehr + S*	+	4, 4, 4	$(SE)_1$	16
S_1	10^{-2}	S180	—	7, 7, 7, 8	S_2	0
S_1	10^{-3}	”	—	All survived†		
S_2	10^0 to 10^{-3}	”	—	All survived		
$(SE)_1$	10^{-3}	Ehr + S	+	4, 4, 4	$(SE)_2$	16
$(SE)_1$	10^{-3}	S180	—	All survived		
$(SE)_1$	10^{-2}	”	—	5, 5, 6	$(SE)_1S_1$	0
$(SE)_1S_1$	10^0 to 10^{-3}	”	—	All survived		
$(SE)_2$	10^{-3}	Ehr + S	+	4, 4, 4	$(SE)_3$	8
$(SE)_2$	10^{-2}	S180	—	6, 6, 6	$(SE)_2S_1$	0
$(SE)_2S_1$	10^0 to 10^{-3}	”	—	All survived		
$(SE)_3$	10^{-3}	Ehr + S	+	3, 4, 4	$(SE)_4$	16
$(SE)_3$	10^{-2}	S180	—	5, 6, 6	$(SE)_3S_1$	0
$(SE)_3S_1$	10^0 to 10^{-3}	”	—	All survived		
$(SE)_4$	10^{-3}	Ehr + S	+	3, 3, 3	$(SE)_5$	32
$(SE)_4$	10^{-2}	S180	—	5, 6, 7	$(SE)_4S_1$	0
$(SE)_4S_1$	10^0 to 10^{-3}	”	—	All survived		
$(SE)_5$	10^{-3}	Ehr + S	+	3, 3, 3	$(SE)_6$	32
$(SE)_5$	10^{-2}	S180	+	4, 4, 4	$(SE)_5S_1$	32
$(SE)_5S_1$	10^0	”	+	3, 3, 3		
$(SE)_5S_1$	10^{-2}	”	+	4, 4, 4	$(SE)_5S_2$	32
$(SE)_5S_2$	10^{-3}	”	+	4, 4, 4	S_3	32
S_3	10^{-3}	”	+	3, 3, 3	S_4	64
S_4	10^{-3}	”	+	3, 3, 3	S_5	64
S_4	10^{-3}	Ehrlich	+	3, 3, 3	S_4E_1	64
S_5	10^{-3}	S180	+	3, 3, 3	S_6	64
S_5	10^{-3}	Ehrlich	+	3, 3, 3	S_5E_1	64

* Ehr + S = mixture of Ehrlich ascites cells and Sarcoma 180 ascites cells.
† All the mice inoculated survived.

supernatant of the infected medium (harvest T1) contained $10^{6.5}$ $LD_{50}/0.1$ ml in mice and 1024 HA units/0.5 ml. Table II shows the subsequent passages in Sarcoma 180 ascites cells *in vitro*. From these results the serial passage of the virus appeared to be established with a stable virus titer but rather variable HA.

3). *Comparative development of CPE in control and infected cells.* "Non-established" strains of Sarcoma 180 ascites tumor cells cannot propagate *in vitro* but survive for several days. The proportion of living cells may be determined at any time by erythrosin staining. Although the viability seems to be rather variable in each case, on the average in our medium 50% of the total cells are alive at 1 day after incubation, 30% at 2 days, 20% at 3 days, 15% at 4 days and 8% at 5 days. Fortunately virus growth in the cells is more rapid than the degeneration of cells themselves, so that the virus infectivity titration can be performed *in vitro* by the criteria of CPE and detection of HA in the infected tubes.

Ten-fold dilutions of the 16th virus passage (T16), from 10^0 to 10^{-8}, were each inoculated into 10 tubes, and percentage of live cells and HA titers were calculated daily from 2 tubes. The cells inoculated with 10^0 to 10^{-3} dilutions were all stained (dead) at 2 days after incubation, the cells inoculated with 10^{-4} to 10^{-6} were all stained at 3 days, while those at 10^{-7} to 10^{-8} were the same as the control (Fig. 1). The stained cells in the infected tubes were more pyknotic and of more irregular shape than those of controls. HA tests also confirmed the CPE (Fig. 2). The infective titer of T16 in mice was also about 10^6 $LD_{50}/0.1$ ml, the same as *in vitro*.

4). *Neutralization and anti-hemagglutination tests with an immune serum prepared against original brain-Columbia SK virus.* With an immune rabbit serum against the original brain-Columbia SK virus possessing an antihemagglutinin titer of 1:1280 and a neu-

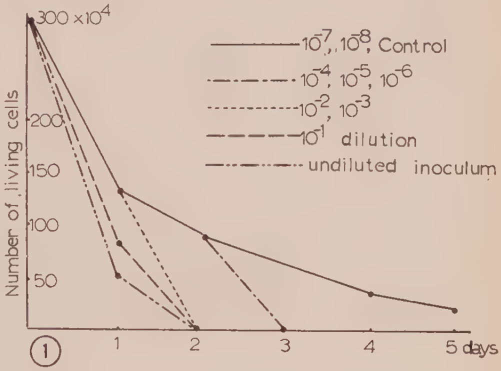
TABLE II. Virus Passage in Sarcoma 180 Ascites Tumor Cells *In Vitro.*

		Harvests				
Inoculum	Dilution	Day	No.	HA titer	In mice LD_{50}	*In vitro* ID_{50}
$(SE)_5S_2$	10^{-1}	3	T1	1024	$10^{6.5}$ per 0.1 ml	
T1	10^{-1}	4	T2	512		
T2	10^{-1}	2	T3	512		
T3	10^{-3}	3	T4	2048	$10^{7.2}$	$10^{7.2}$
T4	10^{-3}	3	T5	512		
T5	10^{-3}	3	T6	512	$10^{6.5}$	
T6	10^{-1}	2	T7	256		
T7	10^{-3}	3	T8	256		
T8	10^{-1}	3	T9	128	$10^{6.5}$	$10^{6.2}$
T9	10^{-1}	3	T10	32		
T10	10^{-2}	3	T11	2		
T11	10^{-2}	3	T12	32		
T12	10^{-2}	3	T13	64	$10^{6.2}$	$10^{6.2}$
T13	10^{-2}	3	T14	512		
T14	10^{-1}	3	T15	256		
T15	10^{-1}	3	T16	512	$10^{6.5}$	$10^{6.5}$
T16	10^{-2}	3	T17	1024		
T17	10^{-2}	2	T18	256		
T18	10^{-2}	3	T19	512	$10^{6.2}$	$10^{6.5}$
T19	10^{-1}	3	T20	256		
T20	10^{-2}	2	T21	1024		
T21	10^{-2}	2	T22	512		
T22	10^{-2}	3	T23	64		
T23	10^{-2}	2	T24	128		$10^{6.5}$
T24	10^{-2}	3	T25	128		
T25	10^{-2}	2	T26	64		
T26	10^{-2}	3	T27	128		

tralizing index of log 4, and the T18 passage of virus, *in vivo* neutralization and anti-hemagglutination tests were performed by usual methods(*2*). The results obtained were the same as those against the brain virus. This indicates that the adapted virus maintains the same antigenicity as the original virus.

5). *Pathogenicity of the adapted virus in mice, albino rats and guinea pigs.* The T18 passage-virus showed about 10^6 LD_{50}/0.1 ml of infectivity when the virus was inoculated intraperitoneally with Sarcoma 180 ascites cells, but only 10^3 LD_{50} without S180 cells. The virus showed no pathogenicity in albino rats, which are susceptible to EMC and Mengo viruses, or in guinea pigs, which are susceptible to brain-Columbia SK virus, even when the undiluted fluid was inoculated intraperitoneally.

Discussion. The purpose of this experiment was to obtain an adapted strain of Columbia SK virus which could be passaged in established cell cultures for *in vitro* screening of antiviral drugs. As reported previously (*2,3*) we had obtained an adapted strain which could propagate in Ehrlich ascites tumor cells *in vivo* with oncolysis, but did not propagate in established Sarcoma 180 cells and HeLa cell cultures *in vitro.* Because there are no established Ehrlich tumor cells which can be cultured serially, we tried to adapt the Ehrlich-adapted Columbia SK virus to Sarcoma 180 ascites tumor cells *in vivo* with a hope that the new strain would then be able to propagate in the "established" or "non-established" S180 cells *in vitro.* It was found that the adaptation of the virus from Ehrlich to S180 ascites cells *in vivo* was possible only through the use of mixtures of Ehrlich and S180 cells. Such an adaptation method has been described by several workers. Sato *et al.*(*6*) reported that an egg-strain of vaccinia virus produced inclusion bodies in Ehrlich ascites cells which were propagating on chick chorioallantoic membranes. Takahashi *et al.*(*7*) also re-



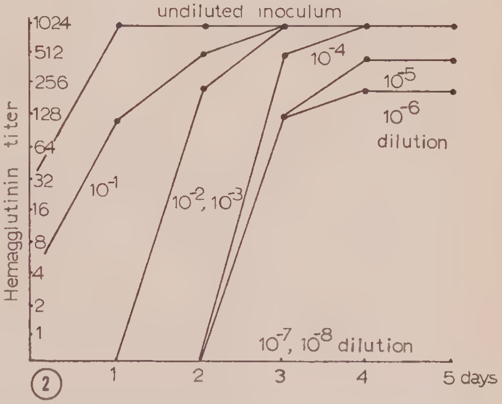


FIG. 1. Cytopathic effect of adapted Columbia SK virus against Sarcoma 180 ascites cells *in vitro.*

FIG. 2. Hemagglutinin of Columbia SK virus in Sarcoma 180 ascites cells *in vitro.*

ported that adaptation of poliovirus type I from HeLa cells to the egg was successful only through inoculation of the virus with HeLa cells but did not succeed with cell-free infected culture fluid. At present it is not certain that our new adapted strain can be cultured serially in established S180 cells. Among the Columbia SK virus group, propagation of Mengo and EMC viruses in mouse ascites cells was reported by Koprowska and Koprowski(8), Flanagan and Colter(9) and Hoskins and Sanders(10). These viruses grew easily from the original brain viruses without necessity of adaptation, but this was not possible with the Columbia SK virus. They did not describe HA in their systems. We have found HA a useful addition to CPE and infectivity in studying the effects of antiviral drugs because it tends to be more specific than CPE and more rapid and quantitative than infectivity titration.

Summary. Adaptation of Columbia SK virus from Ehrlich ascites cells to Sarcoma 180 ascites cells *in vivo*, and the serial passage *in vitro* are described. The adaptation was successful only through the use of mixtures of both cells. The new adapted virus propagates efficiently in Sarcoma 180 ascites cells *in vitro* with CPE and production of HA. This virus-host system has been used for screening tests of antiviral drugs.

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Relation of Potassium and Glucose Release from the Liver in the Unanesthetized Dog.* (26905)

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An interrelationship between potassium and glucose movement has long been noted. Fenn(1) has described the deposition of glycogen in a matrix of potassium and phosphate ions, and has shown that glycogen can not be deposited unless accompanied by appropriate amounts of potassium and water. D'Silva(2) noted transitory increases in plasma potassium with glycogenolysis induced by epinephrine in the intact animal and in the perfused cat liver(3). This has been confirmed(4,5,6,7). However, it has also been demonstrated that epinephrine produces a small increase in blood potassium when the liver is out of the circulation(8,9). Moreover, epinephrine also causes the perfused dog lungs to release potassium(10).

Since glycogen deposition is accompanied by potassium accumulation in the liver, D'Silva(11) suggested that glycogenolysis must result in potassium release from the liver.

In the present study concentrations of potassium and glucose of plasma entering and leaving liver were measured in unanesthetized dogs before and after glucagon and epinephrine administration. Concentration differences across the organ were multiplied by the simultaneously measured hepatic plasma flow rate to give an approximation of net

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rate of hepatic potassium and glucose uptake or output.

Methods. Sixteen experiments were carried out in mongrel dogs whose hepatic vessels were catheterized with large plastic tubing by a previously described technic(12), 2 to 7 days prior to the experiment. All dogs were studied in an unanesthetized state without sedation after an overnight fast. Arterial, portal and hepatic venous blood was withdrawn by a constant withdrawal pump so that 4 to 6 contemporaneous, time-integrated control samples were obtained over 5- to 10-minute intervals. After rapid intravenous administration of glucagon 0.02 mg/kg or epinephrine 1 μg/kg into a systemic vein, samples were obtained at 10-second intervals for 2 minutes and at less frequent intervals thereafter.

Hepatic plasma flow was measured by a modified bromsulphalein method(13). Plasma potassium was determined by an internal standard flame photometer and plasma glucose by the Somogyi-Nelson method(14). Hepatic output of potassium or glucose was calculated as product of concentration differences (hepatic venous minus portal venous concentrations) and hepatic plasma flow rate.

Results. A. *Effect of glucagon.* 1. *Hepatic venous, arterial and portal venous potassium concentration.* The hepatic potassium response to glucagon occurs rapidly. At 20 to 30 seconds after glucagon injection elevation of hepatic venous plasma potassium began. This elevation usually became maximal between 1 and 2 minutes after injection and returned toward control values within 5 to 10 minutes. At its maximum the mean elevation in hepatic venous plasma potassium was 1.2 meq/l above control (Fig. 1).

Lesser increases of arterial and portal venous plasma potassium occurred immediately after hepatic venous elevations.

2. *Hepatic-portal venous plasma potassium concentration difference.* The hepatic-portal venous concentration difference increased from a mean control value of 0.1 meq/l to a mean maximum of 0.7 meq/l after glucagon administration (Fig. 1).

3. *Hepatic potassium output.* Mean control hepatic potassium output was .08 meq/min. There was a markedly increased rate of potassium output from the liver after glucagon administration. Between 1 and 1.7 meq of potassium were released by the liver in the first 2 minutes after glucagon. This potassium release roughly paralleled the increased potassium concentration difference across the organ (Fig. 1).

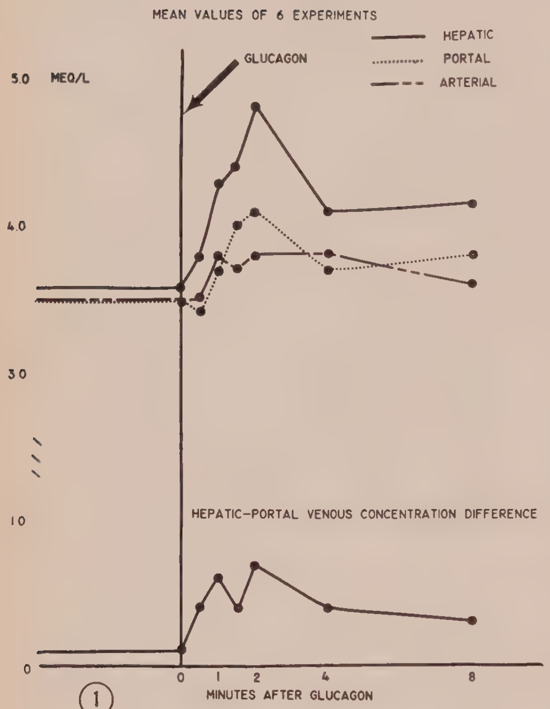
4. *Late potassium movements.* During the ensuing hour there was a gradual fall of potassium in arterial, hepatic venous and portal venous plasma. Hepatic-portal venous concentration difference decreased. The initial output of potassium by the liver gradually fell to become negative; *i.e.*, hepatic potassium uptake.

B. *Effect of epinephrine.* 1. *Hepatic venous, arterial and portal venous plasma concentrations.* Hepatic venous plasma potassium concentrations increased markedly and rapidly after epinephrine administration. The pattern was similar to that following injection of glucagon except that the epinephrine response was shorter in duration. In most instances, return to normal or near normal levels occurred within 120 seconds. Fig. 2 illustrates a representative experiment.

2. *Hepatic-portal venous plasma concentration difference.* The potassium concentration difference across the liver was markedly increased after epinephrine injection. Following this increased positive gradient, a pronounced negative gradient was consistently observed.

3. *Hepatic potassium output.* Rate of hepatic potassium output is represented as a function of time after intravenous epinephrine injection (Fig. 3). Except for being more rapid, the pattern of potassium output from the liver after epinephrine was similar to that following glucagon administration. A total of .4 to .8 meq potassium was released by the liver after administration of epinephrine, 1 μg/kg. Hepatic uptake of a similar amount of potassium followed. An increased hepatic plasma flow began during or immediately after onset of hepatic potassium output.

4. *Hepatic glucose output.* Concentration of glucose in arterial, portal and hepatic ve-



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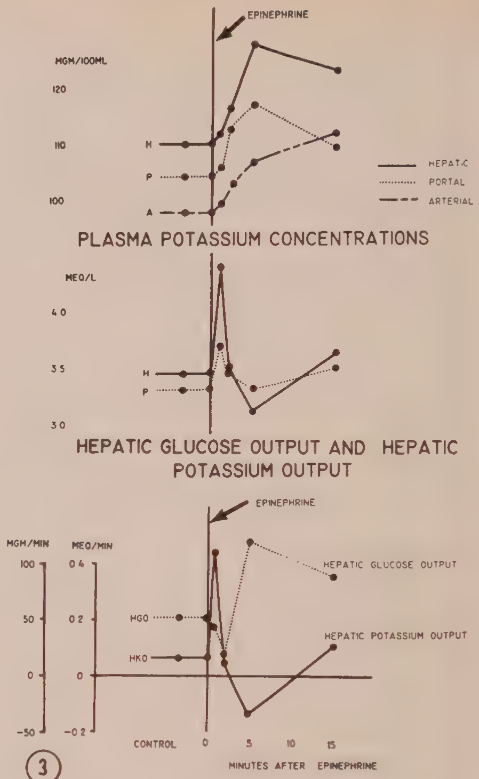

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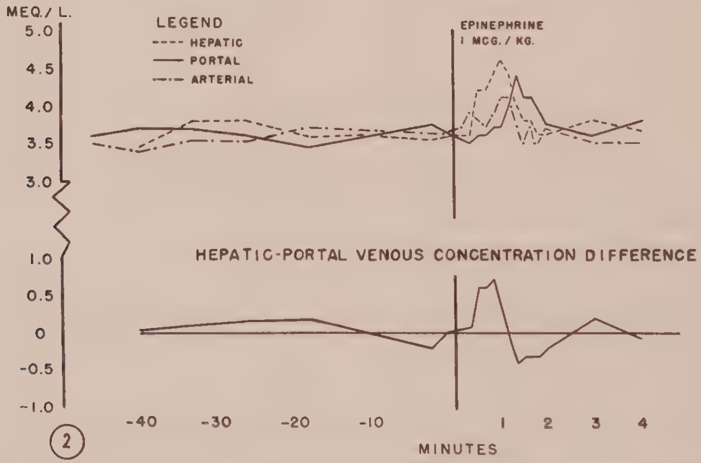

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FIG. 1. Arterial, portal and hepatic venous plasma potassium concentrations and hepatic-portal venous concentration differences of a series of 6 dogs before and after glucagon, .02 mg/kg. There is a marked and rapid rise in hepatic venous potassium levels and in hepatic-portal venous concentration differences appearing within 30 sec. and reaching a maximum 2 min. after inj.

FIG. 2. Arterial, portal and hepatic venous plasma potassium concentrations and hepatic-portal venous concentration differences before and after epinephrine, 1 μg/kg. The pattern of potassium flux is similar but more rapid than that occurring after glucagon administration.

FIG. 3. Concentrations of plasma potassium and glucose, and net flux or output, in a representative experiment in which epinephrine was administered. There is a marked output of potassium prior to an increase in hepatic glucose output.

nous plasma as well as rate of hepatic glucose output are compared with concomitantly measured potassium values in a representative experiment (Fig. 3). Release of potassium by liver, preceded the earliest increase in glucose output.

Discussion. Measurement of rates of net movement of potassium and glucose as a function of time after epinephrine or glucagon injection indicates the sequence of events occurring with hormone-induced glycogenolysis. Potassium is first released by the liver. An increased hepatic glucose output follows 3 to 6 minutes after epinephrine injection. We(15) have previously reported that maximum hepatic glucose output occurred 10 to 20 minutes after glucagon administration.

Of further interest is the observation reported from this laboratory that hepatic blood flow increased after glucagon(16) and epinephrine(17) injection. Hepatic blood flow increases occurred during or immediately after the increased hepatic potassium output following administration of epinephrine. Hepatic blood flow response to glucagon, however, appeared to parallel the increased rate of hepatic glucose output.

The findings of this study refocus attention on the mechanism of glucose release, and strongly suggest that potassium does not merely passively accompany glucose, water and phosphate ion in mass departure from the intracellular phase. Rather, the potassium release is an action well antecedent to that of glucose release, and therefore would appear to be involved in reactions earlier in the glycogenolytic sequence than the actual release of glucose.

Sutherland and coworkers(18) have demonstrated some of the mechanisms whereby glucagon and epinephrine affect transformation of inactive phosphorylase to active phosphorylase. Since muscle phosphorylase has been shown to be potassium dependent, it seems possible that an ionic movement such as that observed in this study may be involved in the primary action of these hormones upon hepatic glycogenolytic enzyme systems.

Summary. The time course of hepatic metabolic and hemodynamic events after hormone-induced glycogenolysis is described in the unanesthetized dog. Increased concentration of hepatic venous plasma potassium and rate of hepatic potassium output occurred after intravenous administration of glucagon, .02 mg/kg. These effects were noted within 20 to 30 seconds after injection and became maximal within 2 minutes. Increased rates of hepatic blood flow and hepatic glucose output occurred 10 to 20 minutes after glucagon administration. A similar but more rapid pattern was observed after intravenous administration of epinephrine, 1 μg/kg. Maximum hepatic venous plasma potassium and hepatic potassium output was reached at about 1 minute after injection. Increased hepatic blood flow occurred during or shortly after increased hepatic venous potassium concentration. Increased rate of hepatic glucose output did not begin until after the potassium efflux from the liver. Hepatic glucose output reached a maximum 3 to 6 minutes after epinephrine injection. The possible significance of potassium efflux in the sequence of glycogenolytic reactions is discussed.

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The Red Blood Cell: An Essential Component of the Toxicity of Strangulation Intestinal Obstruction. (26906)

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It has been demonstrated by several investigators that the peritoneal exudate from dogs which have a strangulated loop of intestine is lethal when given to normal animals(1,2). Such fluid is not always fatal and we have attempted to find out why.

Our data from many dogs and rats revealed that 2 elements were constantly present in any strangulation fluid that was subsequently lethal to normal recipients: an *E. coli* level of at least 10^6 bacteria/ml and a hemogobin level of at least 3 g %. It did not appear that the presence of other bacteria regardless of concentration made any detectable difference. It did appear, however, that if the *E. coli* level fell below that stated, or if the hemoglobin level fell below 3 g % the fluid began to lose its lethal effect. The following experiments were designed to test this hypothesis.

Methods. If the above hypothesis is correct it should be possible to manufacture this lethal fluid in the laboratory. The following groups of experiments were done and the results checked by intraperitoneal injection of the fluid into the rat in a dose of 5 ml/kg. This is the same quantity of fluid used in our earlier experiments. All studies were carried out in male rats of the Sprague-Dawley strain and weighing 200-250 g.

I. The first group of 45 rats were given 5 ml/kg of a 24-hour broth culture of *E. coli*. The inoculum was adjusted to contain 1 × 10^8 organisms/ml.

II. The second group of 15 rats was given whole blood intraperitoneally 5 ml/kg.

III. The third group of 29 rats was given an intraperitoneal injection of *E. coli* plus whole blood. *E. coli* were grown in nutrient broth for 24 hours in presence of added whole blood so that hemoglobin concentration was at least 4 g %.

IV. The fourth group of 40 rats was given an I.P. injection of 10^8 *E. coli* grown in nutrient broth to which had been added rat plasma. The plasma was that amount present in the blood with a hemoglobin concentration of 4 g %. The amount of whole blood necessary to achieve this level was determined, then centrifuged and the resultant supernate added to the broth culture.

V. The fifth group of 174 rats received an I.P. injection of *E. coli* (10^8) in nutrient broth to which 4 g % of hemoglobin had been added in the form of washed red cells (red blood cells were separated by centrifugation and washed 3 times with saline solution).

VI. The final group of 10 rats were given an I.P. injection identical to group V except that *Clostridia welchii* replaced *E. coli*.

The number of bacteria injected was estimated in a Coleman Jr. spectrophotometer at 660 mμ. The actual number of viable bacteria injected was determined by subsequent dilution and plating of an aliquot of the injected culture. Quantitative counts were performed in a Quebec colony counter at 24 hours.

Hemoglobin level was determined using the cyanmethemoglobin technique read in a Klett colorimeter using a 420 filter.

Results. The results are summarized in Table I. When *E. coli* or the hemoglobin solutions were given independently of each other there was no lethal effect. If the 2

TABLE I. Results of Injection of Bacteria and Hemoglobin Concentrations into the Rat Peritoneal Cavity.

Group	No. rats	Bact. conc.	Media	No. killed	%
I	45	*E. coli* 10^8	Nutrient broth	0	0
II	23	None	*Idem* + Hgb 4 g %	0	0
III	29	*E. coli* 10^8	” + whole blood (Hgb 4 g %)	23	79
IV	40	*E. coli* 10^8	” + plasma	0	0
V	174	*E. coli* 10^8	” + washed RBC 4 g %	164	94
VI	10	Clostridia 10^7	” + washed RBC 4 g %	0	0

were incubated together the material became highly lethal. When plasma was used in place of hemoglobin the material was nonlethal (Group IV) and if bacteria other than *E. coli* were used, the material was nonlethal (Group VI). Subsequent experiments have shown that red cells from different species are equally effective in that the same highly toxic effects have been duplicated using washed dog red cells and washed human red cells.

Discussion. Our interest in development of a lethal fluid in the laboratory resulted from the fact that we were unable to produce a uniformly fatal fluid when short loop strangulation obstruction was produced in either the dog or the rat. If several fluids were pooled the resultant pool was usually fatal, but when individual fluids were used the recipient animals were killed in only 50% of instances.

A careful study was made of the quantitative bacteriology and chemistry of the fluid removed from the animals with a strangulating intestinal obstruction. The only two elements of all those measured which appeared to be consistent were the hemoglobin concentration and the level of *E. coli*. The experiments presented here confirm this hypothesis.

The evidence presented cannot be explained on the basis of data presently available. The possibilities are: a) the bacteria act on the red cell to release some factor which is lethal to the animal. b) The red cell in some way interferes with the animal's natural defense mechanism and permits the bacteria to be lethal. (In other words the red cell may act as an adjuvant similar to gum tragacanth(3) or mucin.) c) The bacteria and red cell interact to produce endotoxin. It is possible that the blood group substances which are muco polysaccharides are involved in this reaction. Studies are now under way utilizing various fractions of the red cell in an attempt to elucidate this mechanism.

Summary. The production in the laboratory of a fluid which simulates the "toxic" or lethal fluid of strangulating intestinal obstruction has been accomplished. This fluid requires a hemoglobin concentration of 4 g % and a level of *E. coli* of 10^6 organisms/ml or more. The fluid when injected into normal animals in a dose of 5 ml/kg intraperitoneally is almost uniformly fatal. The mechanism of death appears to be identical with that which follows intraperitoneal injection of raw strangulation fluid.

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Influence of Thyroactive Compounds on Serum and Liver Cholesterol in Rats.* (26907)

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The effects of thyroid hormones on cholesterol metabolism have recently been reviewed (1) and the chemistry and physiology of these hormones have been discussed by Pitt-Rivers and Tata(2). There is now convincing evidence that compounds which affect thyroid function may exert an extrathyroidal effect on cholesterol metabolism. Cutting, Rytand and Tainter(3) demonstrated that effects on basal metabolic rate (BMR) were dissociated from effects on serum cholesterol levels; and more recently Duncan and Best (4) and Boyd(5) have shown that thiouracil exerts an effect on cholesterol metabolism in the athyreotic rat.

There have recently become available a wide variety of thyroxine analogs in which the distribution of nuclear iodine atoms or the structure of configuration of the side chain has been altered. These compounds are being tested in many laboratories to ascertain whether they exert an effect on cholesterol metabolism that is divorced from other thyroid hormone effects. Recent publications(6-10) have described the effects of representative members of this growing series of thyroxine analogs on the serum and liver lipids, heart weights and oxygen consumption of normal and cholesterol-fed rats. Boyd(11) has reviewed the more recent studies with the iodothyronines.

This report deals with the effects of 3 thyroactive compounds: 3,5,3′-triiodothyropropionic acid (Triprop), D-thyroxine (DT_4) and L-thyroxine (LT_4). Actually, this report covers 2 series of experiments, a comparison of 2 dosage levels of Triprop and a comparison of D- and L-thyroxine. The experiments were carried out simultaneously and both series were compared with the same set of controls. Most studies compare the effects of thyroactive compounds upon rats fed either normal or cholesterol-rich diets. We have studied the 3 compounds listed above in rats fed diets high in fat with and without added cholesterol and cholic acid.

Materials and methods. Groups of male Wistar rats, 100-120 g, were maintained for 3 weeks on the diets outlined in Table I. Rats were given daily subcutaneous injections of 2 concentrations of Triprop (0.03 mg/100 g or 0.15 mg/100 g body weight), DT_4 (0.05 mg/100 g body weight) and LT_4 (0.005 mg/100 g body weight. Solutions of the thyroactive compounds were prepared by suspending the compound to be used in a small amount of 95% ethanol, adjusting the pH to 10.0-10.5 with 0.1 N NaOH and diluting with distilled water so that the proper dosage could be administered in 0.10 ml. Fresh solutions were prepared each week. A water-ethanol blank, pH 10.0, was used for

TABLE I. Diets Fed to Rats on Thyroactive Drugs.

	Fat diet	Fat-cholesterol diet
Lard	25	25
Casein	20	20
Salt mix*	4	4
Dextrose	50.5	48
Choline chloride	0.5	0.5
Cholesterol†	—	2.0
Cholic acid	—	0.5

* Salt Mix #2, USP XIII (Nutritional Biochemicals Corp.): Calcium biphosphate (13.58), calcium lactate (32.70), ferric citrate (2.97), magnesium sulfate (13.70), dibasic potassium phosphate (23.98), sodium acid phosphate (8.72), sodium chloride (4.35).

† Lard contained 1% cholesterol, therefore fat diet contained approximately 0.25% cholesterol and fat-cholesterol diet about 2.25%.

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* We wish to thank the Warner-Lambert Research Institute for generous gifts of Triprop, Flint Eaton Division of Baxter Laboratories, Inc. for D- and L-thyroxine and Dr. S. Hier, Wilson and Co., for cholesterol. We are indebted to Miss Shirley Tepper for skillful technical assistance. This work was supported by grants from Nat. Institutes of Health, USPHS, John A. Hartford Foundation, Inc. and Warner-Lambert Research Institute.

TABLE II. Effect of Thyroactive Compounds on Serum and Liver Cholesterol Levels of Rats Fed Fat or Fat-Cholesterol Diets.

	Fat diet					Fat-cholesterol diet				
Group*	No.†	Avg wt gain (g)	Avg liver wt (g)	Avg liver cholesterol (mg/g)	Avg serum cholesterol (mg %)	No.†	Avg wt gain (g)	Avg liver wt (g)	Avg liver cholesterol (mg/g)	Avg serum cholesterol (mg %)
Exp. 1										
T3P-3	4/5	58	8.79	5.22 ± .80‡	99.0 ± 7.4	5/5	34	9.67	56.0 ±14.6	197.0 ± 22.9
T3P-15	5/5	25	6.80	3.63 ± .40	110.1 ±10.9	5/5	26	7.72	43.6 ± 6.6	227.0 ± 35.4
C	5/5	54	9.07	2.96 ± .32	132.0 ±33.9	5/5	61	12.20	45.4 ± 3.6	185.0 ± 20.3
Exp. 2										
T3P-3	3/5	44	10.82	1.72 ± .55	54.8 ± 3.0	4/5	42	10.20	16.3 ± 3.2	80.9 ± 3.3
T3P-15	4/5	13	7.73	3.12 ± .34	51.7 ± 1.0	2/5	14	7.83	21.1	68.6
C	3/5	80	8.68	4.24 ±1.32	68.3 ± 3.4	4/5	46	9.68	29.2 ± 4.6	128.0 ± 8.9
DT4	5/5	–4	6.55	3.10 ± .14	64.1 ± 3.5	3/5	29	8.80	14.4 ± 1.6	78.3 ± 3.7
LT4	3/5	2	7.62	4.10 ± .24	60.8 ± .5	3/5	12	7.51	34.6 ± 1.6	78.2 ± 5.0
Exp. 3										
T3P-3	9/10	– 6	5.27	Lost	83.2 ± 6.8	9/10	—	6.85	Lost	178.7 ± 20.4
T3P-15	7/10	–20	5.53	”	99.1 ± 9.7	6/10	–23	6.74	”	168.0 ± 12.8
C	10/10	22	5.61	”	70.6 ± 4.5	10/10	14	7.47	”	368.5 ± 26.2
DT4	9/10	– 8	5.10	”	82.0 ± 7.6	9/10	–15	6.66	”	157.2 ± 14.5
LT4	8/10	–11	5.07	”	79.1 ± 4.0	10/10	– 1	7.10	”	110.3 ± 11.4
Exp. 4										
T3P-3	10/10	113	11.41	17.90 ±1.7	141.9 ±12.5	10/10	114	13.27	50.1 ± 2.9	184.9 ± 19.8
T3P-15	9/10	57	8.59	7.89 ±1.2	152.7 ±11.2	10/10	66	11.30	58.7 ± 2.1	161.0 ± 18.2
C	9/10	109	12.29	12.80 ±1.4	126.7 ±16.3	10/10	114	13.00	35.4 ± 3.5	206.1 ± 24.2
DT4	10/10	105	12.24	7.23 ±1.9	126.1 ± 7.9	10/10	116	13.31	34.1 ± 3.0	203.7 ± 12.6
LT4	10/10	113	11.13	7.33 ±1.0	143.1 ± 7.8	9/10	114	13.32	33.1 ± 3.4	176.5 ± 12.9
Exp. 5										
T3P-3	3/6	65	9.05	3.97 ± .23	70.0 ± 3.6	6/6	24	11.55	36.2 ± 7.7	69.0 ± 9.2
T3P-15	4/6	13	7.72	4.55 ± .47	65.3 ± 5.3	3/6	–23	8.07	32.9 ± 4.9	128.0 ± 21.9
C	6/6	42	9.43	4.75 ± .34	56.0 ± 5.4	4/6	31	12.40	37.7 ± 2.5	79.5 ± 7.7
DT4	6/6	26	7.75	4.44 ± .40	52.0 ± 9.9	5/6	7	9.79	21.0 ± 6.6	60.0 ± 11.6
LT4	6/6	21	8.32	10.00 ±3.01	49.0 ± 4.3	6/6	18	11.24	36.4 ± 7.4	53.6 ± 13.7

* T3P-3 — Triprop, .03 mg/100 g/day
T3P-15— Triprop, .15 mg/100 g/day
DT4 — D-Thyroxine, .05 mg/100 g/day
LT4 — L-Thyroxine, .005 mg/100 g/day
C — Control

† No. survivors/No. starters

‡ Stand. error

the control rats. After 3 weeks the rats were killed and their blood taken for analysis. Livers were weighed and homogenized in chloroform-methanol 2:1. The liver extract was dried over sodium sulfate and an aliquot used for cholesterol assay. Cholesterol analyses were carried out by the Trinder method (12). Five separate experiments were carried out.

Results and discussion. The results are summarized in Table II. The 5 experiments were carried out during April, July, December, June and October respectively, of a 2-year period. The livers of all the animals in the third experiment were lost in a laboratory accident.

In the experiments involving the high fat diet, the thyroactive compounds had little consistent effect on serum or liver cholesterol values. This was true for the 2 Triprop groups as well as for those on D- or L-thyroxine. On the high fat-high cholesterol diet, administration of Triprop generally lowered serum cholesterol levels; liver cholesterols were also lower in these groups than in the controls. The 2 thyroxine treated groups also showed generally lower serum and liver cholesterol levels than were observed in the controls. At the dosages administered serum cholesterol levels were lower in the L-thyroxine group while liver cholesterol levels were lower in the D-thyroxine groups.

The thyroid state of the rat does not appear to affect total body cholesterol(13,14). We attempted to calculate the total serum-liver cholesterol pool to ascertain if this value might not offer more evidence concerning the effects of the various treatments we were studying. Total liver cholesterol was calculated by multiplying the average liver weights by average liver cholesterol values. The serum cholesterol pool was calculated on the assumption that the average blood volume of the rat is 6% of his body weight(15). Assuming 50% of the blood to be serum, this volume, usually between 4 and 6 ml, was multiplied by the serum cholesterol concentration (mg/ml). The serum cholesterol contribution to the liver-serum cholesterol pool is almost negligible for the groups fed the fat-cholesterol diet, varying between 0.5% and 2% of the pool. Average values are summarized in Table III. There was no consistency in these values. When liver weight and body weight are considered, as they are in these calculations, lower serum or even liver concentrations do not always reflect lower serum-liver cholesterol content.

TABLE III. Average Liver-Serum Cholesterol Pool Size (mg) of Rats Fed Fat and Fat-Cholesterol Diets and Treated with Thyroactive Compounds.

Group*	Fat diet	Fat-cholesterol diet
Exp. 1		
T3P-3	34.1	551.4
T3P-15	29.6	347.8
C	34.1	564.1
Exp. 2		
T3P-3	21.6	169.8
T3P-15	26.4	167.8
C	41.1	288.8
DT4	23.0	130.5
LT4	34.0	264.2
Exp. 4		
T3P-3	214.2	677.8
T3P-15	76.2	672.2
C	151.4	474.6
DT4	97.7	468.2
LT4	99.7	453.3
Exp. 5		
T3P-3	40.5	421.9
T3P-15	38.4	270.0
C	47.9	471.9
DT4	37.0	208.3
LT4	85.7	411.8

* See legends—Table I.

Ruegamer(7) found that in rats fed a high fat diet, Triprop treatment tended to lower serum cholesterol values but neither Triprop (0.03 mg/100 g) or L-thyroxine (0.04 mg/100 g) significantly altered liver cholesterol levels. Duncan and Best(6) found that L-thyroxine (0.0003 mg/100 g) caused a slight increase in serum cholesterol levels and a slight drop in total liver cholesterol; neither were significant. In a longer term experiment, the feeding of L-thyroxine (0.0009 mg/100 g or 0.0006 mg/100 g) lowered serum cholesterol levels while raising liver cholesterol content.

Our experiments indicate that thyroactive drugs can affect serum and liver cholesterol levels in rats fed high fat or high fat-high cholesterol diets. It is of importance to consider the total body cholesterol content of these animals. Clearly, there are no standard data in the literature regarding the effects of thyroactive compounds on cholesterol metabolism, since dosages used, routes, and duration of administration have varied from study to study.

Summary. In a series of 5 separate experiments, rats were fed high fat and high fat-high cholesterol diets and were treated with various thyroactive compounds. Two dosages of 3,5,3′-triiodothyropropionic acid (Triprop) (0.03 mg and 0.15 mg/100 g body weight) were administered subcutaneously and, in general, were found to lower serum and liver cholesterol levels when compared with controls. Administration of D-thyroxine (0.05 mg/100 g body weight) and L-thyroxine (0.005 mg/100 g) also lowered serum and liver cholesterol levels. Among individual experiments there was a variation in magnitude, and in a few instances in the direction, of effect.

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Decline in Rate of Cholesterol Synthesis During Maturation of Chicken Aorta.[*] (26908)

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While most of the cholesterol of the normal artery wall appears to arrive there by transfer from plasma, there has been reason to believe that local synthesis may contribute significantly(1). The experiments described here indicate, among other things, that cholesterol synthesis by avian aortic tissue falls off rapidly as the animal matures.

Materials and methods. White Leghorn cockerels, maintained on a commercial feed, were sacrificed by exsanguination. Aortas were removed, immersed in chilled buffer, freed of clots and connective tissue, and opened longitudinally. In some experiments the aorta was cut into thoracic and abdominal segments, and each of these was split longitudinally into 2 portions, one for incubation with acetate as substrate and the other with mevalonate. Each artery or piece of artery was weighed and placed into an incubation vessel containing 3.0 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), 0.1 ml of 5% dextrose, and 0.1 ml of either sodium acetate-1-

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[†] Most of this work was performed while the author was a Clinical Investigator of the Veterans Admin.

TABLE I. Sterol Synthesis from Acetate and Mevalonate by Chicken Aorta.

			Acetate as substrate					Mevalonate as substrate				
			Before bromination				Loss of radioactivity* in bromination (whole aorta), %	Before bromination				Loss of radioactivity* in bromination (whole aorta), %
			Cholesterol radioactivity, cpm/g tissue			% conversion (whole aorta)		Cholesterol radioactivity, cpm/g tissue			% conversion (whole aorta)	
Exp. No.	Individual body wt, g	Mean aortic wt, g	Thoracic	Abdominal	Whole†			Thoracic	Abdominal	Whole†		
1	1565 1460	1.048	268	933	611	.0158	—	252	187	219	.0269	96
2	1563 1410 1402	.871	319	166	241	.0055	76	298	180	228	.0221	92
3	1560 1363 1330	.639	732	455	595	.0098	—	173	217	192	.0137	—
4	1075 803 725	.568	341	2180	1325	.0173	85	283	355	326	.0230	98
5	547 506 504	.455	—	—	—	—	—	—	—	650	.1410	94
6	495 490 480	.491	—	—	1087	.0519	73	—	—	—	—	—
7	548 412 405	.451	—	—	—	—	—	—	—	1313	.2810	95
8	501 338 311	.369	—	—	4320	.1550	79	—	—	—	—	—
9	395 345 345	.443	—	—	—	—	—	—	—	672	.1420	96
10	385 383 380	.465	—	—	2170	.0977	83	—	—	—	—	—

In Exp. 1-4, each flask contained half of either a thoracic or abdominal aorta. In Exp. 5-10, each flask contained a whole aorta. In all experiments, cholesterol digitonide was isolated from the pooled aortic tissue of the 2 or 3 animals in the group.

* Corrected for losses of material (by weight) during bromination and recovery.

† In calculation of these figures for Exp. 1-4, correction was made for inequality in the sizes of the 4 ''quarters'' of each aorta. Hence, the figure is not identical to the mean of the thoracic and abdominal aorta radioactivities.

TABLE II. Production of $C^{14}O_2$ from Labeled Acetate and Mevalonate by Chicken Aorta.

		Acetate as substrate				Mevalonate as substrate			
		CO_2 radioactivity, cpm/g aorta ($\times 10^{-5}$)			% conversion (whole aorta)	CO_2 radioactivity, cpm/g aorta			% conversion (whole aorta)
Exp. No.*	Wt of aorta, g	Thoracic	Abdominal	Whole†		Thoracic	Abdominal	Whole†	
1	.963	3.33	2.65	2.98	7.9	797	1564	1203	.113
	1.132	1.73	2.11	1.94	4.8	555	1783	1176	.183
4	.498	5.34	5.03	5.19	7.2	3440	2510	2950	.168
	.589	2.86	4.14	3.45	6.0	2330	2310	2320	.123
	.590	2.90	2.03	2.46	3.4	1021	1027	1025	.077
5	.464	—	—	—	—	—	—	265	.058
	.460	—	—	—	—	—	—	374	.079
	.442	—	—	—	—	—	—	579	.123
6	.486	—	—	1.46	7.0	—	—	—	—
	.522	—	—	1.05	5.3	—	—	—	—
	.464	—	—	.93	4.1	—	—	—	—

In Exp. 1 and 4, each flask contained half of either a thoracic or abdominal aorta. In Exp. 5 and 6, each flask contained a whole aorta.

* Numbers correspond to those in Table I.

† In calculation of these figures for Exp. 1 and 4, correction was made for inequality in the sizes of the 4 "quarters" of each aorta. Hence, the figure is not identical to the mean of the thoracic and abdominal aorta radioactivities.

C^{14} (3.7 μc) or sodium DL-mevalonate-2-C^{14} (0.76 μc). Specific activity of both labeled compounds was 1 μc/μmole. Tissues were shaken for 3 hours at 37°C in an atmosphere of 95% O_2, 5% CO_2.

In some experiments, center well flasks were used to permit collection of CO_2. Each flask was stoppered with a serum vial cap, and flushed with the O_2-CO_2 mixture at the start of incubation *via* hypodermic needles inserted through the cap. At the end of incubation, 0.3 ml of 2 N NaOH was delivered into the center well through a long needle, followed by 0.1 ml of 10 N H_2SO_4 into the outer compartment. The flasks were shaken 10 minutes and then unstoppered. The solution in the center well was removed and added to 9-10 ml of 1 N NaOH, which was used to wash the container. To each sample were added 5 ml of 1 M $BaCl_2$. After standing 15 minutes, the precipitated $BaCO_3$ was centrifuged off and washed twice with water and twice with methanol.

Aortas or quarter-aortas from 2 or 3 animals were pooled in each experiment. The non-saponifiable material was obtained(2), and to it were added 12 mg of pure cholesterol as carrier. An aliquot of this solution was used for direct precipitation of cholesterol digitonide(2). The cholesterol in another aliquot was purified *via* the dibromide (3), and digitonide was prepared from this purified material.

Samples of $BaCO_3$ and cholesterol digitonide were mounted by filtration onto tared filter paper discs. Each sample was dried and weighed, and counted in an ultra-thin-window flow gas counter (Nuclear-Chicago Model D47) having an efficiency of approximately 30%. A minimum of 2,000 counts was recorded for each sample. All radioactivity data were corrected for self-absorption to a standard thickness of 5 mg/cm².

Results and discussion. These experiments reveal a striking diminution in the rate of aortic sterol synthesis with increasing body weight (Table I). This was observed with both acetate and mevalonate as substrate. The relationship to body weight was apparent whether the sterol precursors of cholesterol were included (non-brominated material) or removed by bromination. The decrease in sterol synthesis with increasing body weight and aortic size was not attributable to impermeability of the thicker aortas to substrate, inasmuch as production of $C^{14}O_2$ failed to exhibit a similar trend (Table II). Plots of the data suggest that there may be

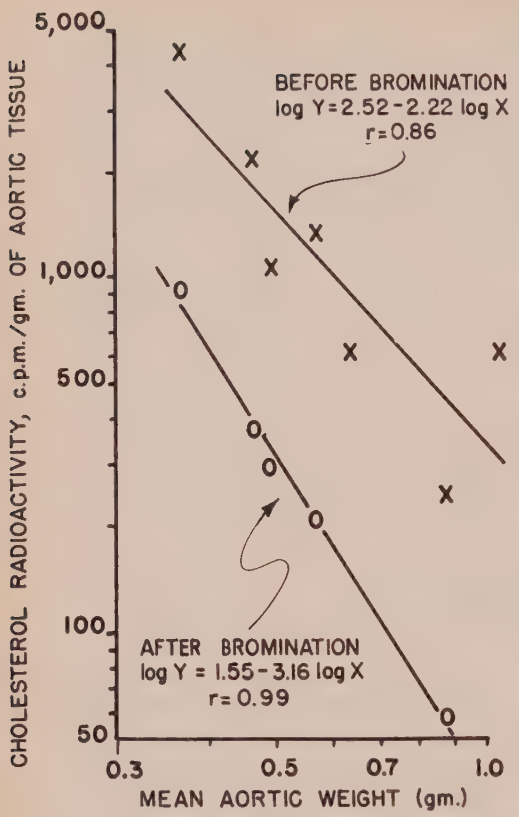

FIG. 1. Sterol synthesis from acetate by chicken aorta.

an inverse linear relationship between log cholesterol synthesis rate (c.p.m./g of tissue) and either log aortic weight or log body weight. This is particularly striking with regard to the data of the acetate experiments, using log aortic weight as abscissa (Fig. 1). A log-log plot for the mevalonate experiments (not shown in the figure) is less suggestive of a linear relationship, although a correlation coefficient of 0.85 is obtained between log cholesterol synthesis rate (c.p.m./g of tissue) and log aortic weight, using the data from the non-brominated samples.

The basis of these relationships is not completely elucidated by the present experiments. However, it seems likely that cholesterol synthesis in aortic tissue is largely related to tissue growth. It is also possible that the decrease in cholesterol synthesis during maturation may be a reflection of decreasing tissue cellularity. The data of the present experiments do not permit a definitive test of these possibilities.

The thoracic and abdominal aortic segments exhibit no consistent differences in rates of cholesterol synthesis, despite distinct differences in wall structure and in susceptibility to atherosclerosis(4).

As in other experimental systems(5), mevalonate was a more efficient precursor of sterol, and a poorer source of CO_2 carbon, than was acetate (Tables I and II). However, efficiency of sterol synthesis from mevalonate was still quite low—only about twice that observed with acetate—and almost all the radioactivity was removed by bromination. The presence of practically all the sterol radioactivity in the high-counting cholesterol precursors implies that the experiment was terminated relatively early, in comparison to the situation in the acetate experiment. However, existing information on cholesterol biosynthesis suggests that there is only one pathway for incorporation of acetate carbon into sterol. and that mevalonate or a closely related compound is an obligatory intermediate(5). These observations may be reconciled by postulating that aortic tissue is less permeable to mevalonate than to acetate, or that for other reasons mevalonate has slow access to cholesterol-synthesizing enzymes, and that sterol synthesis from mevalonate is consequently delayed in this *in vitro* system.

Summary. *In vitro* synthesis of labeled cholesterol by aortic tissue of cockerels was found to fall off rapidly with maturation, with either acetate-1-C^{14} or mevalonate-2-C^{14} as precursor. Production of $C^{14}O_2$ failed to show a similar relationship, indicating that the declining rate of cholesterol synthesis is not a consequence of decreasing permeability of the tissue to the substrate. In the system employed, mevalonate was only twice as efficient as acetate as a source of sterol carbon, and almost all the sterol radioactivity derived from mevalonate was removed by bromination. Thoracic and abdominal aorta displayed similar rates of cholesterol synthesis.

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Hepatic Clearance of Endotoxin Absorbed from the Intestine.[*] (26909)

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Endotoxin absorbed from the gut has been demonstrated in the blood of animals in 3 types of traumatic shock(1). In the normal animal the endotoxin is extracted from the blood and destroyed almost immediately by the reticulo-endothelial system (R.E.S.)(2). But in the shocked animal the capacity of the system to extract or destroy endotoxin is severely impaired(2). Thus endotoxin becomes free to injure the circulation and to prevent recovery. The amount of endotoxin absorbed varies with the size of the pool of the gram-negative flora in the gut(3). Additional evidence to this effect is presented below.

Method. New Zealand rabbits (average wt 2 kg) with normal intestinal flora, as indicated in each instance by culture from rectal swabs, were anesthetized with nembutal intravenously, and the abdomen opened with aseptic precautions through a midline incision. The intestine was gently exteriorized, and 7-10 ml of blood withdrawn from the portal vein into heparinized tubes. Similarly, blood was withdrawn from the inferior vena cava. These bloods were cultured and were found to be uniformly free of bacteria. They were also tested for the presence of endotoxin by the method of Smith and Thomas(4). A positive test consisted of multiple hemorrhages and death of at least 4 of 6 ten-day-old chick embryos, to each of which 0.1 ml of the same sample of blood had been administered. Death of not more than 2 of 6 embryos was considered evidence of the absence of endotoxin. Because the cultures from rectal swabs do not always accurately reflect the pattern of the intraintestinal flora, cultures from the lumen of ileum and colon were obtained at the end of each experiments as a check upon the preoperative cultural data from rectal swabs.

Five groups of experiments were performed. In Group 1 the procedure as described above was carried out. In Group 2 the procedure was the same, except that 3 hours preoperatively 100 mg of an *E. coli* $0111B_4$ endotoxin (MLD/100 = 3-4 mg/kg) in 30 ml of normal saline was given by gavage. In Group 3 the procedure was the same as in Group 2, but in addition 2.75 ml/kg of Thorotrast was given intravenously when the endotoxin was fed. In Group 4 the procedure was the same as in Group 1, except that the rabbits used were coliform-free by the cultural data from preoperative rectal swabs. In Group 5 the procedure was the same as in Group 4, except that these rabbits were given neomycin (250 mg/kg) by gavage daily for 3 successive days, and again 3 hours preoperatively on the 4th day.

Results are listed in Table I. *Group 1.* The data of Group 1 indicate the presence of endotoxin in the blood of the portal vein in one-half, and in the blood of the systemic veins in one-sixth of rabbits with a normal intestinal flora. There was no instance in

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TABLE I. Assay of Blood for Endotoxin by Chick Embryo Test.

Group	No.	Positive portal vein blood	Positive inf. vena cava blood
		%	%
1. Normal coli-positive	30	50	17
2. Normal coli-positive + endotoxin by gavage	10	60	30
3. Normal coli-positive + Thorotrast	9	100	89
4. Coli-neg.*	10	40	0
5. Coli-neg. + preoperative neomycin	28	16	4

* Coli-negative signifies absence of coliform bacteria in cultures of preoperative rectal swabs. Cultures of the lumen of ileum and colon at time of laparotomy showed that of the 10 rabbits only 4 were, in fact, coli-negative. The number of coliforms in the other 6 was very small, however.

this or any other group in which the blood of a systemic vein was toxic when the blood of the portal vein was non-toxic.

Comment. These data are evidence of absorption of endotoxin from the intestine. They are consistent with previous data obtained by direct perfusion of the spleen with endotoxin, showing that an intact R.E.S. can extract some 80% of the endotoxin presented to it(2). This latter observation would allow for some toxin in the blood leaving the R.E.S. Hence the occasional presence of toxicity in systemic vein blood (17%) of normal rabbits is to be expected. Indeed the escape of endotoxin into the general circulation may account for occasional variations from the normal responses to states of stress in which endotoxin can become a critical determinant. *E. g.*, in any given series of animals exposed to severe hemorhagic shock, a few animals develop irreversibility to transfusion very much sooner than the majority. Contrariwise, some 10-15% do not develop irreversibility because of a better than normal detoxifying capacity of the R.E.S.(5). Animals with a low pool of endotoxin in the gut because of a coliform-free intestinal flora also do not develop irreversibility to transfusion(6). In them one would expect most of the bloods from the portal as well as those from the systemic vein to be non-toxic (See data of Group 4 below). But even when the flora is normal, absence of toxicity might be expected when the amount of endotoxin entering the portal vein at the time of sampling is less than can be detected by the chick embryo test. One can thus account for the fact that one half the samples in Group 1 were non-toxic.

Group 2: Comment. The data of Group 2 show a higher incidence of toxicity in the systemic blood than was found in Group 1, suggesting that an increase in size of the pool of endotoxin in the gut can increase the amount absorbed so as occasionally to overtax the liver's extracting capacity.

Group 3: Comment. The data of Group 3 demonstrate that Thorotrast eliminates the liver's capacity for extracting the endotoxin delivered to it from the intestine, confirming a previous observation by Cremer and Watson(7) and supporting some of our own later observations(6). This phenomenon doubtless accounts for the enormous increase in sensitivity to endotoxin shown by Thorotrast-pretreated animals when given endotoxin for eliciting the Shwartzman reaction. Such a spontaneously generated endotoxemia may explain the lethal property of Thorotrast when given alone in large doses(6).

Group 4: Comment. The data of this group require interpretation in the light of the cultural data obtained postmortem, showing that of the 10 rabbits considered coliform-free before operation, only 4 were coliform-free by direct culture of the ileum and colon. Of these 4, one yielded a positive portal vein blood. The remaining 6 showed some coliform bacteria in the ileum and colon, and of these 6, three yielded positive portal vein bloods. It is probable that the zero incidence of toxicity in the systemic vein blood of these 10 rabbits is due to the very much smaller pool of endotoxin in this group than in those with a normal coliform flora.

Group 5: Comment. The nearly total elimination of toxicity in portal as well as systemic blood as a result of giving neomycin to a group of coliform-free rabbits is strong support for the view that antibiotics given orally prevent endotoxemia, and therefore, irreversibility to transfusion in severe and prolonged hemorrhagic shock as a result of sharply reducing the size of the intraintestinal pool of endotoxin(8). Thus it is clear that not only can the size of the intraintestinal pool be readily altered, but also that the amount of endotoxin which enters the circulation varies with the size of the pool.

Discussion. These observations supplement considerable published evidence that endotoxin is continuously absorbed from the intestine(9), and that a number of phenomena are influenced by this process in a hitherto unsuspected manner(6). Thus in 1957 we confirmed previous observations that mortality from a lethal dose of endotoxin is reduced by prior or simultaneous administration of an antibiotic. This was also the case when a non-absorbable antibiotic was given(10). From these data we concluded that an MLD/100 of endotoxin is not lethal *per se,* but by virtue of the participation of additional endotoxin absorbed from the gut following injection of the "lethal" dose. The endotoxin which is continuously absorbed from the gut may also account for the occurrence of the generalized Shwartzman reaction after a single dose of endotoxin in the pregnant animal (11,12), and for the sporadic occurrence of the local Shwartzman reaction in mice following a single dose of endotoxin(13). This is all the more likely in view of the observation that tetracycline given orally eliminates such responses until the intestinal flora acquire resistance to the antibiotic(14). The apparently wayward responses of rabbits, tested under comparable circumstances, to administration of 2 spaced doses of endotoxin for the purpose of inducing the generalized Shwartzman reaction may also find its explanation in variations in the size of the intraintestinal pool of endotoxin, and in the rate of its absorption into the circulation. That rabbits with a coliform-free intestinal flora subjected to hemorrhagic shock do not develop the endotoxemia observed in coliform-bearing rabbits(15), and the recent report of Schaedler and Dubos(16) that pathogen-free Swiss mice are more resistant to endotoxin than mice of the same breed bearing coliform bacteria, further support the hypothesis that the pattern of the intestinal flora, tolerance to endotoxin, and tolerance to traumatic shock are interrelated.

Summary. Data are presented showing that endotoxins absorbed from the intestine are present in the blood, and in much higher incidence in portal than in systemic vein blood. The incidence of endotoxemia in portal vein blood varies with the size of the intraintestinal pool of endotoxin. A reduced capacity of the R.E. system in the liver for extracting endotoxin allows endotoxin in the portal vein blood to traverse the liver and enter the general circulation.

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Metabolism of 5-Hydroxy-D- and L-Tryptophan in Man: Synthesis of Serotonin from the D-Isomer. (26910)

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It is generally assumed that formation of 5-hydroxytryptamine (serotonin) and 5-hydroxyindoleacetic acid (5HIAA) from 5-hydroxy-DL-tryptophan (5HTP) is due only to metabolism of the L-isomer. However, the actual fate of the D-isomer has not been determined. It is important that this be ascertained since the 5HTP which is available commercially is the racemic mixture of its D- and L-isomers, and thus is the only form which has been used for pharmacologic and biochemical studies in man. It has been shown that D-5HTP is not a substrate for aromatic L-amino acid decarboxylase(1), and could not therefore serve as an immediate precursor of serotonin. However, the theoretical possibility existed that formation of 5HIAA from the D-isomer might be effected by D-amino acid oxidase *via* the intermediate, 5-hydroxyindolepyruvic acid. The resolution of 5HTP into its optical antipodes by Morris and Armstrong(2) afforded the opportunity to study this possibility.

Quite unexpectedly, it was found that infusion of D-5HTP into human subjects resulted in increased urinary excretion of not only 5HIAA, but also serotonin. In this investigation the 5-hydroxyindole metabolites of both D- and L-5HTP have been determined following infusion of each isomer separately, and the urinary serotonin formed from D-5HTP has been identified chemically.

Methods. The 2 subjects of the study were 23 years of age, a male normal volunteer and a female patient with very mild uncomplicated primary hypertension; both were hospitalized at the Clinical Center of Nat. Inst. of Health. All fruit and fruit juices were excluded from their diets.

Small amounts of the D- and L-isomers of 5HTP were made available through the generosity of Dr. Marvin D. Armstrong, Fels Research Institute, Yellow Springs, Ohio. DL-5HTP was purchased from a commercial source. After being heated in the powder form at 80°C for 45 minutes, the various forms of 5HTP were dissolved in 150 ml of 0.9% NaCl and infused intravenously at a constant rate over periods of 150 minutes in subject B.W. and 75 minutes in L.M. Urine was collected for 12 hours after beginning each infusion. Urines for control assays were collected for 12 hours following infusion of 150 ml of 0.9% NaCl and also during corresponding 12-hour periods in which there were no infusions.

Urinary L-5HTP was measured by fluorometric determination of the serotonin formed after incubation with aromatic L-amino acid decarboxylase(3). Serotonin was separated from the urine on a weakly acidic cation exchange resin and measured fluorometrically (4). Urinary 5HIAA was measured by colorimetric determination of its 1-nitroso-2-naphthol complex(5). Two-dimensional chromatography of the urine was performed as described by Jepson(6).

The purity of the D- and L-5HTP was established initially by Morris and Armstrong (2) and was confirmed in this laboratory as follows: These compounds were chromatographed in a 2 dimensional system(6), and only one Ehrlich reacting spot was present in each case. To demonstrate that it was entirely free of L-5HTP, the D-isomer was incubated with partially purified aromatic L-amino acid decarboxylase from guinea pig kidney(7). While L-5HTP was nearly quantitatively converted to serotonin by this enzyme, no serotonin was formed from the D-5HTP.

Results. Quantification of 5-hydroxyindole metabolites. The amounts of L-5HTP, serotonin and 5HIAA excreted in the 12 hours following separate infusions of D-, L- and DL-5HTP are presented in Table I, and are compared with corresponding values from control periods. Following infusion of 60 mg of D-5HTP, there was an average increment in 5HIAA excretion of 2.5 mg/12 hr. Further-

TABLE I. Excretion of 5-Hydroxyindoles Following Infusion of the Racemic Mixture (DL-) and Separate D- and L-Isomers of 5HTP.

		Urinary excretion of 5-OH-indoles		
Subject	Infusion	L-5HTP	Serotonin	5HIAA
		mg/12 hr		
B.W.	.9% NaCl	—	<.2	1.4
	None	<.5	<.2	2.3
	"	—	<.2	1.2
	D-5HTP 60 mg	<.5	1.3	4.5
	D-5HTP 60 "	—	1.5	4.3
	L-5HTP 30 "	3.2	5.2	13.5
	DL-5HTP 60 "	3.0	5.5	12.1
L.M.	.9% NaCl	—	<.2	3.4
	None	<.5	<.2	3.0
	D-5HTP 60 mg	<.5	1.8	5.4
	L-5HTP 30 "	3.3	4.8	14.4
	DL-5HTP 60 "	3.5	6.6	19.2

more, excretion of serotonin was increased from scarcely measurable amounts (<0.2 mg/12 hr) to an average of 1.57 mg/12 hr. It is apparent, therefore, that conversion to serotonin and 5HIAA represents a definite though small pathway of metabolism of D-5HTP, accounting for approximately 8% (on a molar basis) of the infused dose. No measurable L-5HTP was excreted after infusion of the D-isomer. Semiquantitative estimation of 5HTP on 2-dimensional chromatography indicated that most of the D-amino acid (70-90%) was excreted into the urine unchanged. No indole metabolites other than these mentioned were seen on chromatography of the urine following D-5HTP.

In contrast, 59-65% (on a molar basis) of L-5HTP was excreted as the sum of 5HIAA and serotonin. Of the 0.136 mM of L-5HTP infused, an average of 11% was excreted as L-5HTP, 21% as serotonin, and 42% as 5HIAA. It may also be seen that most of the serotonin and 5HIAA excreted following infusion of DL-5HTP is formed from the L-isomer.

Identification of serotonin excreted after infusion of D-5HTP. To characterize the apparent serotonin chemically, several samples of urine collected after D-5HTP infusion were carried through the column separation step of the serotonin assay(4). The eluates of these columns were pooled, and carried through a butanol extraction procedure(5) to purify the apparent serotonin further. Authentic serotonin was carried through the ion exchange and extraction steps in the same manner. The fluorescence properties of apparent serotonin were determined on a 1 ml aliquot of the final acid extract, to which 0.3 ml of 12 N HCl had been added. Both the excitation and the fluorescence spectra were found to be indistinguishable from those of authentic serotonin. The wavelengths of excitation and fluorescence maxima were 300 mμ and 540 mμ, respectively (wavelengths uncorrected).

For chromatographic characterization, an aliquot of the acid extract was adjusted to a neutral pH, evaporated under a jet of nitrogen, and desalted with ethanol: acetone (5:1). This was evaporated under a nitrogen jet to 0.2 ml, which was then applied to Whatman #1 chromatographic paper alongside a sample of authentic serotonin. An ascending chromatogram was developed with n-butanol: glacial acetic acid: H_2O (120:30:50) and dipped in Ehrlich's reagent. Ehrlich reacting spots with identical blue colors and identical Rf values (0.52) appeared at the sites of authentic and apparent serotonin.

Discussion. The metabolism of most of the infused L-5HTP to serotonin and 5HIAA was predictable from previous studies on this isomer *in vitro* and in animals(1). The appearance of appreciable quantities of serotonin in the urine after infusion of D-5HTP was certainly not expected, however, for it had not been demonstrated previously that this D-amino acid could be metabolized to serotonin. Since no serotonin was formed when D-5HTP was incubated *in vitro* with aromatic L-amino acid decarboxylase, it would not appear that serotonin is synthesized directly from the D-amino acid by action of this enzyme. Probably, formation of serotonin *in vivo* requires conversion of the D-5HTP to its L-antipode. In studying the inversion of D-phenylaminobutyric acid to its enantiomorph, duVigneaud *et al.*(8) demonstrated that the keto acid was an intermediate in such an inversion. Therefore, it is likely that inversion to L-5HTP would begin with metabolism of D-5HTP to 5-hydroxyindolepyruvic acid, a reaction catalyzed by the flavoprotein enzyme, D-amino acid oxidase. While some 5-hydroxyindolepyruvic acid may be converted directly to 5HIAA, this keto acid intermediate also could be metabolized to L-5HTP by transamination, a reaction in which 5HTP is known to participate(9).

The only mammalian tissues known to contain D-amino acid oxidase are liver and kidney(10), and the kidney has by far the greater concentration of the enzyme. Thus, it is likely that much of the conversion of D-5HTP to its L-antipode took place in renal tissue. Since the kidney is also a rich source of L-aromatic amino acid decarboxylase, it seems likely that this organ is the chief site of the conversion of D-5HTP to serotonin. This would be compatible with the observation that D-5HTP has no pharmacologic effect on the central nervous system(1) where there is little or no D-amino acid oxidase. If further studies confirm that conversion of D-5HTP to serotonin occurs chiefly in the kidney, the use of this D-amino acid may provide an interesting approach to investigating the direct effect of serotonin on renal mechanisms.

Although constituting a relatively minor pathway, metabolism of D-5HTP to serotonin and 5HIAA should certainly be borne in mind in studies involving the D-isomer or DL-racemic 5HTP. Furthermore, the evidence that D-5HTP is converted to its L-antipode in humans probably does not represent a phenomenon specific for this amino acid. It is known that many D-amino acids may be substituted for their enantiomorphs in the diets of animals(11), and that the D-isomers of several non-physiologic amino acids may be inverted to their L-antipodes *in vivo*(8,12). All of these findings suggest that some conversion to an L-antipode may be a common pathway in the metabolism of many D-amino acids in man.

Summary. As anticipated, the metabolism of 5-hydroxy-L-tryptophan infused in humans was found to result in excretion in the urine of approximately 20% of the infused dose as serotonin and 40% as 5-hydroxyindoleacetic acid. It was quite unexpected, however, to find that conversion to serotonin and 5-hydroxyindoleacetic acid also represents a definite though small (8%) pathway for metabolism of 5-hydroxy-D-tryptophan. The significance of the latter observation is discussed and the suggestion made that serotonin synthesis from the D-amino acid probably occurs *via* formation of its antipode through the action of D-amino acid oxidase and transamination.

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Threshold and Pattern of Electroshock Seizures after 250 r Whole-Body X-Irradiation in Rats.* (26911)

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Moderate doses of X-irradiation modify the responses of nervous tissue to electrical stimulation(1,2). Exposure to 500 r whole-body X-irradiation in rats lowers the threshold to electroshock convulsions(3-6), alters the pattern of tonic-clonic seizures and delays the recovery of normal brain function after a maximal convulsion(4-6). Furthermore, the changes in convulsive reactivity persist for at least 180 days after a single exposure to 500 r X-irradiation(5,6).

At present these functional changes cannot be explained by morphological alterations because previous work has indicated that doses above 1000 r are necessary in order to affect cellular morphology in the central nervous system(7). It is possible that systemic and local metabolic alterations following X-irradiation could be responsible for these effects on brain function. For example, body growth is retarded after 500 r X-irradiation. However, the reduction in body weight alone can not explain the lower than normal threshold(6). The effects of exposure to low to moderate X-ray doses on brain function have not been clearly defined nor are the underlying mechanisms completely understood in terms of the available data.

The present study was undertaken to compare the effects of 2 doses (250 and 500 r) of X-irradiation on electroshock convulsions for a period of 20 days post-irradiation in rats.

Materials and methods. Animals. Thirty adult male Long-Evans rats of about 240 g body weight at the beginning of the experiment were divided into 3 groups of 8-13 animals each as follows: (1) control, electroshocked; (2) 250 r, electroshocked; (3) 500 r, electroshocked. Food (Purina Fox Chow Pellets) and water were given *ad libitum* except for a 12-hour period of fasting prior to irradiation.

Body weights were recorded periodically for all animals throughout the experiment.

X-irradiation. The experimental animals were exposed to single doses of whole-body irradiation at 13 r/minute with a 250 kv 15 ma X-ray machine (filters: 0.5 mm Cu and 1.0 mm Al).

Electroshock technics. The apparatus for producing electroshock seizures in rats is a constant-current stimulator delivering a sinusoidal 60 CPS current. Woodbury and Davenport(8) have described this stimulator and the various electroshock methods in detail. The following methods are used in this experiment: 1. *minimal electroshock seizure threshold (EST) test,* as a measure of brain excitability; 2. *maximal electroshock seizure (MES) pattern test,* as a measure of spread of excitation; and 3. *return of the righting reflex (RRR) test,* as a measure of brain recovery processes.

The EST is defined as the minimal amount of alternating-current in milliamperes (ma) delivered for 0.2 second through corneal electrodes which will elicit just barely detectable clonic convulsions of ears, jaws, and forepaws without loss of equilibrium and lasting only a few seconds. The MES is produced by delivering 150 ma for 0.2 second through corneal electrodes. With this supramaximal stimulus, normal rats show typical maximal (tonic-clonic) convulsions. Total duration of the seizure, duration of the tonic phase with its flexor and extensor components and duration of the clonus are determined for each animal. The RRR test measures the duration of the depression period

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TABLE I. Effect of 250 r and 500 r Whole Body X-Irradiation on Body Weight of Rats.

		Body wt, g						
		Days before irradiation	Days post-irradiation					
Group treatment		4	0	2	5	10	15	20
Control (13)*	Mean	251	238	265	280	289	298	306
	S.E.	7.2	4.4	5.1	6.2	6.6	7.7	4.8
	% change†		–5	6	11	15	19	22
250 r (8)	Mean	246	229	238 §	243 §	250 ‡	260	287
	S.E.	7.8	7.1	9.2	11.9	18.0	22.4	18.3
	% change		–7	–3	–1	2	6	17
500 r (9)	Mean	246	227	232 §	237 §	222 §	241 §	253 §
	S.E.	5.0	4.9	6.0	7.8	13.9	15.0	13.3
	% change		–8	–6	–4	–10	–2	3

* No. of animals in parentheses.
† % change is computed from pre-irradiation value.
‡ t test comparison with controls P ≤.10.
§ ” ” ” ” P ≤.05.

elapsing between the end of the MES and the spontaneous rise of the animal.

In the present experiment the EST was determined 4 times at intervals of 48 hours prior to any experimental manipulation in order to obtain a baseline for all animals. Following fasting and irradiation of groups 2 and 3 and fasting of group 1, EST determinations were made within 5 hours after X-ray exposure, daily for the next 5 days, and then every 48 hours until the end of the experiment on day 20. MES pattern and RRR were assessed on day 5 and 20 post-irradiation.

Results. At 20 days post-irradiation the mortality for the 250 r and 500 r irradiated groups was 1.4% and 2.2%, respectively.

The pattern of body growth indicated by changes in body weight is presented in Table I. Control rats grew at a normal rate characteristic for the age, sex and strain of the animals; it has already been shown that electroshock alone does not alter body growth (6). Irradiation at 500 r significantly (by the t test) retarded the rate of body growth as compared to controls from day 2 to 20 post-irradiation. The difference between body weights of controls and of the 250 r group was statistically significant from day 2 to 10 post-irradiation, *i.e.* for a shorter period of time than after 500 r.

Control rats demonstrated the progressive increase in threshold after the initial fall due to fasting shown to be associated with increasing age(9). After 500 r and 250 r irradiation this normal increase was inhibited, and irradiated rats maintained a lower threshold than non-irradiated controls for the duration of the post-irradiation period (Fig. 1). The difference between irradiated and control rats became statistically significant at about day 8 post-irradiation and remained significant to day 20. There was no difference in threshold between the 2 levels of irradiation.

Irradiation at doses of 250 and 500 r had no significant effect on maximal seizure pat-

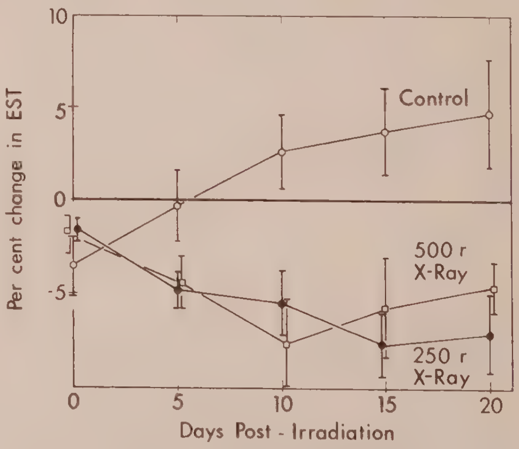

FIG. 1. Changes in electroshock seizure threshold (EST) for a 20-day period for control, 250 r, and 500 r groups. Data are presented as percent of change from a pretreatment value taken at one day before irradiation and/or fasting. Stand. errors are indicated by bracketed lines.

TABLE II. Effect of 250 and 500 r Whole-Body X-Irradiation on Maximal Seizure Pattern of Rats 20 Days after Exposure.

Group and treatment		Maximal seizure pattern (duration of phases in sec.) Tonus	Clonus	Total seizure
Control (12)*	Mean	14.4	32.0	46.5
	S.E.	.35	3.5	3.5
250 r (7)	Mean	13.4	18.9‡	32.3‡
	S.E.	.42	1.8	1.5
500 r (7)	Mean	14.5	23.7†	38.2†
	S.E.	.53	3.2	2.7

* No. of animals in parentheses.

† t test comparison with controls indicated P $\leq .10$.

‡ t test comparison with controls indicated P $\leq .05$.

tern on the 5th day post-irradiation. By day 20 post-irradiation the duration of the total maximal seizures was reduced and this was the result of a shorter clonic phase (Table II). In the present experiment postictal depression was not modified to a statistically significant degree by irradiation. However, the irradiated rats tended to have a longer period of depression after maximal seizures than control animals. This finding has been described previously(6).

Discussion. Single exposures of rats to whole-body X-irradiation in moderate doses can alter the threshold for electrically induced seizures. With the methods used in these experiments, increased convulsability is indicated by a lower than normal threshold. The data presented show that both 500 r and 250 r irradiation inhibit to the same degree the characteristic progressive increase in threshold with age and produce similar changes in the maximal seizure pattern.

It is interesting that there have been a number of studies indicating a plateau in response to a range of radiation doses. Quastler *et al.*(10) showed that a 3½ day mortality for mice was a stable finding in a dose range of 500 to over 4000 r. In a study of abdominal sensitivity to X-ray in rats (Bond *et al.* 11), a plateau was observed in average day of death and in body growth over a dose range of 1600 to 2200 r. Quastler and Baer (12) working with irradiated seeds of Mung beans showed a degree of inhibition of growth which was stable over various dose ranges. Finally in the study of brain electrical activity by Gangloff and Haley mentioned above (1), the same magnitude of change was observed after 200 and after 400 r.

The works cited together with the results of the present experiment suggest that irradiation-induced changes in several systems occur to a maximum extent after a threshold dose of radiation is exceeded. These changes may involve a particular mechanism which manifests an all-or-none response to radiation. Increasing the radiation dose above the threshold level would have no added effect unless or until a second mechanism is affected by the radiation.

In view of the retardation in body growth after irradiation it might be possible to correlate general metabolic alterations with functional changes in the central nervous system. However, evidence has been presented that there is no direct relation between alterations in rate of body growth and changes in threshold after irradiation(6). Similarly in the present experiment (Table I and Fig. 1), the fact that body weight was reduced to a greater extent by the higher radiation dose whereas changes in brain function were of the same magnitude with both doses supports the hypothesis that these phenomena are somewhat independent expressions of selective systemic and local metabolic processes.

The increased excitability after irradiation indicated by the lower than normal thresholds is also apparent from some of the aspects of the maximal seizure pattern (Table II). For example, in all irradiated groups the ratio between lengths of tonus and clonus was greater than in the controls. On the other hand, the shorter total duration of the maximal seizures in the experimental animals as compared to controls suggests interference by irradiation with the spread of nervous impulses in the central nervous system. Shortening of maximal seizures, together with prolongation of the postictal depression could be interpreted as an increase of central inhibition or as a greater than normal degree of fatigue of the neural elements after heightened activity. This interpretation which has been proposed in relation to the effects of 500 r (6) seems to be valid for the smaller dose of 250 r.

Summary. The effects of single doses of 250 and 500 r whole-body X-irradiation on threshold and pattern of electroshock seizures were followed in rats for 20 post-irradiation days. Irradiated rats had lower electroshock seizure thresholds than controls. Total duration of the maximal electroshock seizures was shorter in the irradiated rats than in controls. This was due to a significant shortening of the clonic phase of the seizure. These findings are interpreted as indicating that brain excitability is increased by irradiation at 250 and 500 r. However, heightened seizure activity cannot be sustained as long in irradiated rats as in controls. The magnitude of the changes in brain convulsability was the same for the 2 doses of X-irradiation while the effects on body growth were dose-dependent in this range.

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